ol. 62, 1979

cta Haematologica

ounded 1948 by E. Meulengracht, K. Rohr and G. Rosenow
ontinued by H. Lüdin (1960–1977)

fficial Organ of the European Division of the International Society of Haematology
eneral Secretary J. W. Stewart, London

S. Karger Basel München Paris London New York Sydney



S. Karger AG P O Box, 4009 Basel (Switzerland)

Printed in Switzerland by Buchdruckerei National-Zeitung + Basler Nachrichten AG Basel

Contents Vol. 62, 1979

No. 1

No. 2

No. 5–6

Original Papers

Short Communication

Cultivation of Haematopoetic Stem Cells and of Committed Leukocyte Progenitor Cells
Proceedings of a Symposium held in Marburg a. d. Lahn, March 15, 1979
Editor: W.-D. Gassel and K. Havemann, Marburg a. d. Lahn

Editorial

Acta haemat. 62, 1-3 (1979)

Variants of Vitamin K Dependent Coagulation Factors

Congenital disorders of blood coagulation can be due to the complete absence or decreased concentration of one of the coagulation factors or to the production of an abnormal one. The abnormal inactive coagulation factors are usually referred to as CRM-positive or CRM-reduced variants. CRM is an abbreviation for *material* that shows a *cross-reaction* with an antibody against a specific coagulation factor. True deficiencies as well as abnormal variants of the vitamin K dependent factors have been reported [1, 2, 6, 7]. Usually the variants are detected by means of their reduced coagulant activity; the latter becomes apparent by comparison of the results of functional and immunological assays. The variants can be characterized further by biochemical methods and by their ability to be activated by nonphysiological agents like trypsin, staphylocoagulase, snake venoms and a number of thromboplastins. At the moment it appears that the severity of the bleeding symptoms only correlates with the residual coagulant activity.

The accompanying paper of *Girolami et al.* [this issue] reports on the observation of a pedigree with a genetic variant of factor VII very similar to factor VII Padua. The factor VII Padua, described by the same authors [4], is the first genetic variant of factor VII of which the nature of the underlying defect might be hypothesized. It is highly probable that the mutation has occurred in that part of the molecule that is responsible for its interaction with the tissue factor. Apart from these two (possibly related) families eight other families have been reported to have a variant of factor VII with a strongly reduced coagulant activity.

At present we know 9 defective variants of factor II (Barcelona, Quick, Cardeza, San Juan, Brussels, Padua, Molise, Madrid, Metz), 5 variants of factor X (Friuli a.o.), and a very large number of factor IX variants. During the last 2 years about 180 pedigrees with hemophilia B have been found to have an abnormal factor IX molecule (hemophilia B⁺, CRM-positive, CRM-reduced). Whether the properties of all these factor IX variants are different cannot be established at this moment. Because specific immunologic assays for factors II, VII, IX and X have become widely available, it can be expected that many additional variants will be detected in the near future.

At present detailed knowledge on the mechanism of action of the vitamin K de

pendent coagulation factors becomes available the mechanism of proteolytic activation, the interaction with other coagulation factors (tissue factor factor V factor VIII) the binding of Ca^{++} ions and the affinity for adsorption to phospholipid surfaces This knowledge can now be used to develop simple assays for several functional aspects of these coagulation factors.

Communication on the subject of genetic variants presently is dependent on the use of geographical epitheta like prothrombin Barcelona, factor VII Padua, factor IX Chapel Hill or factor X Friuli. Such a nomenclature will be sufficient as long as the number of variants is relatively small, and their abnormality is clearly defined. However when both the detection rate and the number of so-called CRM positive variants increases, the need for a more adjusted nomenclature becomes apparent (factor VII and especially factor IX variants). In our opinion such a nomenclature should give information on the observed abnormalities of the variant molecule. As long as the only abnormality consists in a reduced specific coagulant activity there is no criterium to distinguish the variants from each other

The majority of the factor II variants has been studied extensively and it appears that variants referred to by different names really represent different abnormal factor II molecules. Most of the variants of factor VII have not been characterized and we consider it appropriate that they have not been given names. However in our opinion the introduction of factor VII Verona is not justified. In contrast to factor VII Padua, factor VII Verona [3] cannot be distinguished by any technic from the other factor VII variants with low specific coagulant activity A reversed situation exists in the field of factor X. The 3 variants described by *Denson et al* [2] in 1970 are clearly different from each other although their characterization might be extended. In our opinion these variants are entitled to have a name. As might be expected some superfluous name giving has started in the field of factor IX variants where, for instance, factor IX Worcester [8] stands for a factor IX molecule without detectable coagulant activity

The factor VII variant introduced as factor VII Padua [4] gives an excellent example of how a very simple experiment (one prothrombin time with a human thromboplastin and one prothrombin time with a bovine thromboplastin) can give valuable information on the nature of the defect. Other tests of comparable simplicity – providing information on for instance Ca^{++} binding capacity affinity for adsorption to phospholipids, or susceptibility to proteolytic activation – may be developed and included in the routine screening of coagulation factor variants. It is our proposal that the results of such assays are used as basis for an appropriate nomenclature. Such a nomenclature can be developed using the initial proposals of the Working Party of *Graham et al.* [5]

R. M Bertina, E. Briët and J J Veltkamp

References

1 Bertina, R. M. and Veltkamp J J.. The abnormal factor IX of hemophilia B variants. Thromb. Haemostasis *40* 335–349 (1978).
2 Denson, K. W E., Lurie, A. De Cataldo, F and Mannucci, P M.. The factor X defect. Recognition of abnormal forms. Br J Haemat. *18* 317–327 (1970).
3 Girolami, A. Falezza, G., Patrassi, G., Stenico M., and Vettore, L.. Factor VII Verona coagulation disorder double heterozygosis

with an abnormal factor VII and heterozygous factor VII deficiency Blood *50*: 603–610 (1977).
4 Girolami, A., Fabris, F Dal Bo Zanon, R., Ghiotto, G and Burul, A.. Factor VII Padua. A congenital coagulation disorder due to an abnormal factor VII with a peculiar activation pattern. J Lab clin. Med. *91* 387 395 (1978).
5 Graham, J B., Barrett, D A. H Bloombäck, B Cann, H. M Hardisty R. M., Larrieu, M. J and Renwick, H. J.: Reports from the International Committee of Haemostasis and Thrombosis. I. A genetic nomenclature for human blood coagulation. Thromb. Diath. haemorrh. *30*. 2–11 (1973).
6 Mazzucconi, M. G Mandelli, F Mariani, G Briët, E., and Veltkamp J J A CRM-positive variant of factor VII deficiency and the detection of heterozygotes with the assay of factor like antigen. Br J Haemat. *36*. 127–135 (1977).
7 Owen, C. A., Jr Henriksen, R. A.; McDuffie, F C., and Mann, K. G.. Prothrombin Quick. A newly identified dysprothrombinemia. Mayo Clin. Proc. *53* 29–33 (1978).
8 Yang, H. C.. Purification and immunological studies of factor IX Worcester Blood *50*: suppl. 1, p. 289 (1977).

Received. December 23, 1978
Accepted: December 27 1978

Hemostasis and Thrombosis Research Unit, University Hospital Leiden, Rijnsburgerweg 10, 2333 AA Leiden (The Netherlands)

Original Papers

Acta haemat. 62: 4–11 (1979)

Another Family with the Factor VII Padua Clotting Defect[1]

A. Girolami, G. Patrassi, G. Cappellato and A. Casonato[2]

University of Padua Medical School, Institute of 'Semeiotica Medica', Padua

Key Words. Factor VII abnormalities · Thromboplastins · Prothrombin complex · Thrombotest · Prothrombin time

Abstract. Another family with the factor VII Padua defect is described. The proposita is a 18-year-old female who presented easy bruising and epistaxis. The main laboratory features of the defect are: prolonged prothrombin time, normal partial thromboplastin time, normal Stypven-cephalin clotting time and normal Thrombotest. Factor VII activity is low when assayed using rabbit brain thromboplastin but is normal when assayed using ox brain thromboplastin. The parents of the proposita were consanguineous (first cousins) and were heterozygotes. Other family members were also shown to be heterozygotes for the abnormality. The present patient is not related to the other family with this disorder. The abnormality may be more widespread than originally thought.

Several congenital coagulation disorders due to abnormal factors have been described during the past years. Several dysfibrinogenemias, a few dysprothrombinemias, hemophilia B_M and B, factor X Friuli, are now well recognized clinical entities [2, 5, 9, 14, 21, 22, 25–27]. We have recently described a family with 2 patients who presented an abnormal factor VII defect [18]. This abnormal factor VII could not interact or could interact only poorly with rabbit brain thromboplastin but could still normally interact with ox brain thromboplastin preparations. As a consequence factor VII resulted to be low (about 9%) when assayed using rabbit brain thromboplastin, whereas it was perfectly normal (100%) when ox brain thromboplastin was included in the assay system. The peculiar factor VII defect was termed 'factor VII Padua'.

The object of the present paper is to report another patient belonging to a different kindred with this peculiar abnormality.

[1] This study was supported in part by a grant from the Ministero Pubblica Istruzione, Rome (grant 1592-77) and by a grant from the Venetian Region.

We wish to express our gratitude to Mr. *G. Boeri* and Mr. *D. Stocco* for valuable technical assistance.

Case Report

The proposita is an 18-year-old female who was sent to us for further investigations because of persistently prolonged prothrombin time. Parents

were consanguineous (first cousins) but family history was negative for bleeding manifestations (fig. 1).

The proposita was first noted to ha easy bruising and occasional epistaxis in childhood. Menstruations are referred to be occasionally abundant. At the age of 17 the patient presented severe epistaxis which required two anterior packings. Altogether bleeding tendency has been mild but so far the patient had no surgical challenges (operations, tooth extractions, etc.).

At the time of study there were no bleeding manifestations and physical examination was negative.

Material and Methods

Material and methods ha been described in *detail in previous papers [7 10, 12, 13 17 18]*

Mixing experiments were carried out by mixing increasing quantities (10 20, 40 and 80%) of patient plasma with normal plasma and carrying out prothrombin time on the mixture [20]. The thromboplastin used in this experiment was rabbit brain and lung preparation containing fibrinogen and factor V 0.25 ml of the thromboplastin preparation was incubated at 37 °C for 5 min and then 0.05 ml of the test plasma or of dilution thereof was added and the clotting time measured. The results in seconds were then plotted against the concentration of patient plasma in the mixture.

The neutralization test as carried out as previously reported [18] using an anti-factor VII antiserum kindly supplied by Dr *H Heimburger* Behringwerke Laboratories, Marburg, FRG. This antiserum is rabbit raised and is only neutralizing one since it fails to yield satisfactory factor VII precipitates in the immunodiffusion, electroimmunoassay or other immunological systems. However in the neutralization test the preparation appears to be specific since it does not inhibit other *clotting factors*.

Results

The results are summarized in tables I–III

Prothrombin time using rabbit brain and lung thromboplastin was moderately prolonged and the same was true for the PP Test and the Normotest. However thrombotest was within normal limits and this was confirmed using several batches of the re-

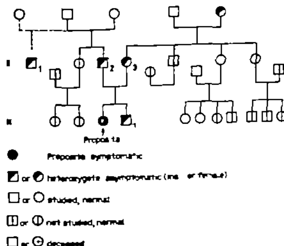


Fig. 1. Family pedigree. The parents of our proposita are first *cousins and are both heterozygous* for the abnormal factor VII. The paternal grandfather and the maternal grandmother of our proposita were brother and sister The paternal grandfather was probably also heterozygote 2 of his sons are heterozygotes for the abnormality in spite of the fact that they were born from different wives.

Original Papers

Acta haemat. 62: 4–11 (1979)

Another Family with the Factor VII Padua Clotting Defect[1]

A. Girolami, G. Patrassi, G. Cappellato and A. Casonato[2]

University of Padua Medical School, Institute of 'Semeiotica Medica', Padua

Key Words. Factor VII abnormalities · Thromboplastins · Prothrombin complex · Thrombotest · Prothrombin time

Abstract. Another family with the factor VII Padua defect is described. The proposita is a 18-year-old female who presented easy bruising and epistaxis. The main laboratory features of the defect are: prolonged prothrombin time, normal partial thromboplastin time, normal Stypven-cephalin clotting time and normal Thrombotest. Factor VII activity is low when assayed using rabbit brain thromboplastin but is normal when assayed using ox brain thromboplastin. The parents of the proposita were consanguineous (first cousins) and were heterozygotes. Other family members were also shown to be heterozygotes for the abnormality. The present patient is not related to the other family with this disorder. The abnormality may be more widespread than originally thought.

Several congenital coagulation disorders due to abnormal factors have been described during the past years. Several dysfibrinogenemias, a few dysprothrombinemias, hemophilia B_M and B+, factor X Friuli, are now well recognized clinical entities [2, 5, 9, 14, 21, 22, 25–27]. We have recently described a family with 2 patients who presented an abnormal factor VII defect [18]. This abnormal factor VII could not interact or could interact only poorly with rabbit brain thromboplastin but could still normally interact with ox brain thromboplastin preparations. As a consequence factor VII resulted to be low (about 9%) when assayed using rabbit brain thromboplastin, whereas it was perfectly normal (100%) when ox brain thromboplastin was included in the assay system. The peculiar factor VII defect was termed 'factor VII Padua'.

The object of the present paper is to report another patient belonging to a different kindred with this peculiar abnormality.

Case Report

The proposita is an 18-year-old female who was sent to us for further investigations because of persistently prolonged prothrombin time. Parents

[1] This study was supported in part by a grant from the Ministero Pubblica Istruzione, Rome (grant 1592-77) and by a grant from the Venetian Region.

[2] We wish to express our gratitude to Mr. *G. Boeri* and Mr. *D. Stocco* for valuable technical assistance.

Table III. Main laboratory features of family members shown to be heterozygotes

Position in family tree	PT (rabbit brain)	PT Test, sec (rabbit brain)	Thrombotest sec	Factor VII activity (rabbit brain), %	Factor VII CRM
I	15.8	31 1	42	60	120
II	16.0	32.6	39	50	90
II (father of proposita)	16.1	33 1	41	55	100
II_1 (mother of proposita)	16.1	33.0	40	52	95
III_1 (brother of proposita)	16.7	33.4	40	45	105
Normal values	14–15 sec	23–27 sec	38–43 sec	80–120	70–130

Table IV Differential diagnosis of known factor VII factor IX (hemophilia B_M), and factor X deficiency abnormalities

Condition	PTT	PT (rabbit brain)	Thrombotest (ox brain)	Stypven-cephalin clotting time	Trypsin clotting time	Comment
Classical factor VII deficiency	N	P	P	N	N	
Classical factor X deficiency	P	P	P	P	P	
Factor VII Padua defect	N	P	N	N	N	
Factor X Friuli defect	P	P	P	N	P	
Hemophilia B_M	P	N (1)	P	N	N (1)	(1) or slightly prolonged

agent. The prothrombin time using another ox brain thromboplastin preparation was also normal. The Stypven-cephalin clotting time and the trypsin clotting time were also normal. No inhibitor was present in the proposita s plasma since normal plasma or normal serum in equal parts fully corrected the prothrombin time. The mixing experiments ruled also out the presence of inhibitors (fig. 2) Thromboplastin generation test, partial thromboplastin time, prothrombin consumption, platelet and vascular tests were all within normal limits. Fibrinogen, fibrinolysis, thrombin time and clot solubility were all normal too. Specific factor assays using a rabbit brain and lung thromboplastin, revealed a factor VII level of 10%/ of normal. Intermediate levels (approximately 32–46%/ of normal) were observed using human (brain or placenta) or simian (brain) tissue thromboplastins. Using an ox brain thromboplastin, factor VII was perfectly normal (100%/). Using a porcine brain thromboplastin, a near normal level was found (65%/) Immunological studies by means of the neutralization test (fig. 3) revealed that our proposita had a normal factor VII antigen. Platelet and vascular tests

Table I. Coagulation study in our proposita

Tests	Results	Normal values
Bleeding time	3 min	< 5 min
Platelet count	200,000	150,000–350,000
Clot retraction	complete after 6 h	complete after 10 h
Prothrombin consumption	> 90%	> 90%
PTT	41.5 sec	32–42 sec
TGT	15 sec in 8 min	≤ 16 sec in 6 or 8 min
PT (Simplastin®)	32 sec	14–15 sec
PT of mixture of proposita plasma + plasma of another case with factor VII Padua	31 sec	
Normotest	100.2 sec	22–26 sec
PP Test	62.5 sec	23–27 sec
Thrombotest	38.5 sec	38–43 sec
Stypven-cephalin clotting time	10.8 sec	10–11 sec
Trypsin clotting time	19 sec	18–20 sec
Thrombin Time	20 sec	18–22 sec
Fibrinogen	300 mg/dl	250–450 mg/dl
Thromboelastogram	normal	
Factor II, V VIII IX, X, XI XII	normal	60–160%
Factor XIII	clot insoluble	clot insoluble
Factor VII	see table II	

Table II. Prothrombin time and factor VII level in our proposita as obtained using different tissue thromboplastins

Thromboplastin	Source	Prothrombin time, sec		Factor VII level, % of normal
		proposita	normal control	
Simplastin, Lot. 0513087 (Warner-Chilcott Laboratories)	rabbit brain and lung	32	14	10
Thromboplastin reagent Lot. ADT78A (Dade Laboratories)	rabbit brain	33	13 7	11
Brain Thromboplastin Lot. 12P645 (Ortho Laboratories)	rabbit brain	34.5	15	9
British comparative thromboplastin batch 047	human brain	20.1	14.5	32
Thromborel Lot. 1147A (Behringwerke Laboratories)	human placenta	19 4	16	40
Calcium-thromboplastin Lot. 187 (Stago-Biochemia Laboratories)	simian brain	18.1	13.5	36
Porcine brain thromboplastin (Stago Laboratories)	porcine brain	43	34.5	65
Ox brain thromboplastin (Nygaard Laboratories)	ox brain	37.5	38	100

Table III. Main laboratory features of family members shown to be heterozygotes

Position in family tree	PT (rabbit brain)	PP Test, sec (rabbit brain)	Thrombotest sec	Factor VII activity (rabbit brain), %	Factor VII CRM
I_1	15.8	31.1	47	60	120
II_1	16.0	32.6	39	50	90
II_4 (father of proposita)	16.1	33.1	41	55	100
II_5 (mother of proposita)	16.1	33.0	40	52	95
III_1 (brother of proposita)	16.7	31.4	40	45	105
Normal values	14–15 sec	23–27 sec	38–43 sec	80–120	70–130

Table IV. Differential diagnosis of known factor VII, factor IX (hemophilia B), and factor X deficiency abnormalities

Condition	PTT	PT (rabbit brain)	Thrombotest (ox brain)	Stypven-cephalin clotting time	Trypsin clotting time	Comment
Classical factor VII deficiency	N	P	P	N	N	
Classical factor X deficiency	P	P	P	P	P	
Factor VII Padua defect	N	P	N	N	N	
Factor X Friuli defect	P	P	P	N	P	
Hemophilia B	P	N (1)	P	N	N (1)	(1) or slightly prolonged

agent. The prothrombin time using another ox brain thromboplastin preparation was also normal. The Stypven-cephalin clotting time and the trypsin clotting time were also normal. No inhibitor was present in the proposita's plasma since normal plasma or normal serum in equal parts fully corrected the prothrombin time. The mixing experiments ruled also out the presence of inhibitors (fig. 2). Thromboplastin generation test, partial thromboplastin time, prothrombin consumption, platelet and vascular tests were all within normal limits. Fibrinogen, fibrinolysis, thrombin time and clot solubility were all normal too. Specific factor assays using a rabbit brain and lung thromboplastin, revealed a factor VII level of 10%/ of normal. Intermediate levels (approximately 32–46%/ of normal) were observed using human (brain or placenta) or simian (brain) tissue thromboplastins. Using an ox bram thromboplastin, factor VII was perfectly normal (100%/). Using a porcine brain thromboplastin, a near normal level was found (65%/). Immunological studies by means of the neutralization test (fig. 3) revealed that our proposita had a normal factor VII antigen. Platelet and vascular tests

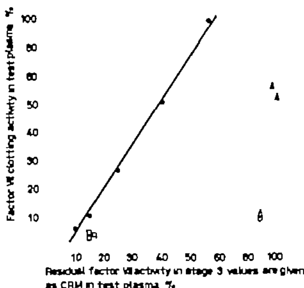


Fig. 2. Neutralization test in the proposita (open triangle) and in her parents (crosses) who are both heterozygotes. The dark triangles and open circles refer to the CRM found in the index patients with this disorder (heterozygotes and homozygotes, respectively). In every instance there is a clear discrepancy between activity in test plasma and residual activity in stage 3 expressed as CRM or antigen. The open squares refer to 3 patients with congenital factor VII 'true' deficiency In this latter case there is no significant discrepancy be tween clotting activity and CRM The dots and the best fit line represent the calibration curve obtained by serial dilutions of pooled normal plasma in the neutralization assay

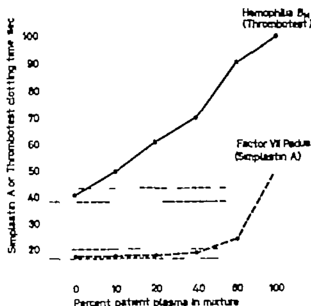


Fig 3 Simplastin A mixing experiment in the proposita. Even 20% of normal plasma present in the mixture is able to correct almost fully the defect. For comparison the curve obtained with Thrombotest in a hemophilia B_M patient is also reported. In the latter case, because of the inhibitor even 10 or 20% of patient plasma is able to prolong significantly the Thrombotest clotting time of the mixture. The broken lines limit the Thrombotest and Simplastin A normal clotting times.

were all within normal limits. The father the mother brother and a few additional members of the maternal and paternal side had a slight prolongation of the prothrombin time and were considered heterozygotes for the abnormal factor VII. In fact, Thrombotest was normal in every instance. Factor VII activity using rabbit brain and lung thromboplastin varied between 45 and 60% but immunological assays revealed a normal factor VII CRM in all these cases (table IV)

Discussion

Congenital factor VII deficiency was first described by *Alexander et al* [1] in 1951 About 100 cases have been described so far in the world literature [12, 13] A correct diagnosis of factor VII deficiency is based on a prolonged prothrombin time together with a normal partial thromboplastin time Stypven-cephalin clotting and trypsin clotting times. A diagnosis of factor VII Padua defect is based on the same criteria together with a normal Thrombotest value. Our patient meets these criteria fully She is homozygous for the abnormality whereas both parents are heterozygotes. The propo-

Table V Tentative classification of factor VII abnormalities on the basis of reported cases.

	Author	Year	Factor VII activity %	Factor VII antigen (CRM) %	Comment
Factor VII+ or factor VII reduced	*Goodnight et al.* [20]	1971	10	35 (1)	(1) value given as residual activity
	Denson et al. [6]	1972	15	50	
			13	40	
			0	18	
	Briët et al. [4]	1977	< 1	20	
	Mazzucconi et al. [24]	1977	1	100	
Factor VII Verona	*Girolami et al.* [17]	1977	20 (2)	55	(2) rabbit or human brain thromboplastin level is higher using ox brain thromboplastin
Factor VII Padua	*Girolami et al.* [18]	1978	9 (3)	100	(3) rabbit brain thromboplastin level is normal using ox brain thromboplastin

Patients with factor VII antigen or CRM level of less than 15 were excluded patients with an activity antigen difference of less than 15% were also excluded.

sita is not known to be related to our previous family with this peculiar disorder but comes from a geographical area close to the valley where the first patients were discovered. Therefore, the possibility of a remote relationship between the two families remains open.

Factor VII Padua appears to be different from the other factor VII abnormality described by us: factor VII Verona. In this latter case, in fact, there is no significant difference between rabbit brain and human brain thromboplastin [17] clotting times. Thrombotest clotting times are shorter than expected [18] but there always remains a discrepancy between factor VII level as determined by ox brain thromboplastin and factor VII cross-reacting material.

The nature of the abnormality present in factor VII Padua is unknown. We know so far only that this abnormal factor VII is absorbable by barium sulfate as normal factor VII. It is likely that the defect consist of an abnormal reactivity' of the site of interaction with rabbit brain thromboplastin.

The identification of this peculiar factor VII defect has complicated the differential diagnosis of serum prothrombin complex factors. Till a few years ago it was thought that factor VII could be differentiated from a factor X defect on the basis of a normal Stypven-cephalin clotting time in the former.

In 1970, with the discovery of factor X Friuli [9] this was shown to be untenable. Stypven-cephalin time, in fact, is normal in the Friuli abnormality. If one takes into account that even a hemophilia B variant,

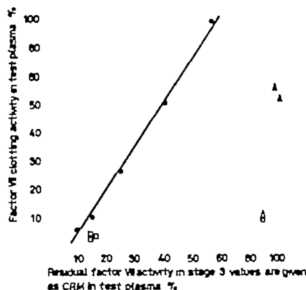


Fig. 2. Neutralization test in the proposita (open triangle) and in her parents (crosses) who are both heterozygotes. The dark triangles and open circles refer to the CRM found in the index patients with this disorder (heterozygotes and homozygotes, respectively) In every instance there is a clear discrepancy between activity in test plasma and residual activity in stage 3 expressed as CRM *or antigen. The open squares refer to 3 patients* with congenital factor VII 'true deficiency In this latter case there is no significant discrepancy between clotting activity and CRM. The dots and the best fit line represent the calibration curve obtained by serial dilutions of pooled normal plasma *in the neutralization assay*

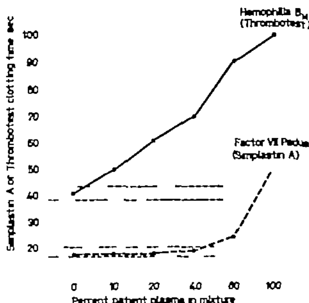


Fig. 3 Simplastin A mixing experiment in the proposita. Even 20% of normal plasma present in the mixture is able to correct almost fully the defect. For comparison the curve obtained with Thrombotest in a hemophilia B_M patient is also reported. In the latter case, because of the inhibitor even 10 or 20% of patient plasma is able to prolong significantly the Thrombotest clotting time of the mixture. The broken lines limit the Thrombotest and Simplastin A normal clotting times.

were all within normal limits. The father the mother brother and a few additional members of the maternal and paternal side had a slight prolongation of the prothrombin time and were considered heterozygotes for the abnormal factor VII In fact, Thrombotest was normal in every instance Factor VII activity using rabbit brain and lung thromboplastin varied between 45 and 60% but immunological assays revealed a normal factor VII CRM in all these cases (table IV)

Discussion

Congenital factor VII deficiency was first described by *Alexander et al.* [1] in 1951. About 100 cases have been described so far in the world literature [12, 13] A correct diagnosis of factor VII deficiency is based on a prolonged prothrombin time together with a normal partial thromboplastin time Stypven-cephalin clotting and trypsin clotting times. A *diagnosis of factor VII* Padua defect is based on the same criteria together with a normal Thrombotest value. Our patient meets these criteria *fully* She is homozygous for the abnormality whereas both parents are heterozygotes. The propo-

Congenital coagulation disorder due to an abnormal factor VII. J. Lab. clin. Med. 91 387–395 (1978).
19 Girolami, A., Patrassi, G. M., and Calla, G. Thrombotest mixing experiments in congenital coagulation disorders of the prothrombin complex and in coumarin treated patients. An additional evidence against the presence of an inhibition in the botter. Folia haemat. 104 463–470 (1977).
20 Goodnight, S. H., Feinstein, D. I. Osterud, B., and Rapaport, S. I. Factor VII antibody-neutralization material in hereditary and acquired factor VII deficiency. Blood 37 1–8 (1971).
21 Hougie, C. and Twomey J. J. Hemophilia B. A new type of factor IX deficiency. Lancet i: 698–700 (1967).
22 Josso, F. Monasterio de Sanchez, J. Lavergne, J. M., Menaché, D., and Soulier J. P. Congenital abnormality of the prothrombin molecule (factor II) in four siblings. Prothrombin Barcelona. Blood 38. 9–16 (1971).
23 Levanon, M., Rimon, S., Shani, M., Ramot, S., and Golberg, E. Active and inactive factor VII in Dubin-Johnson syndrome with factor VII deficiency and on coumadin administration. Br. J Haemat. 23 669–677 (1972).
24 Mazzucconi, M. G., Mandelli, F. Mariani, G., Briët, E., and Veltkamp J. J.: A CRM-positive variant of factor VII deficiency and the detection of heterozygotes with the assay of factor like antigen. Br. J. Haemat. 36. 127–135 (1977).
25 Menaché, A. Constitutional and familiar abnormal fibrinogen. Thromb. Diath. haemorrh., suppl. 13 pp. 173–185 (1964).
26 Shapiro, S. S. Martinez, J. and Holburn, R. R. Congenital dysprothrombinemia. An inherited structural abnormality. J. clin. Invest. 42. 2251–2259 (1969).
27 Shapiro, S. S. Prothrombin San Juan. A complex new dysprothrombinemia. In Hemker and Veltkamp, Prothrombin and related factors, pp. 205–212 (Leiden University Press, Leiden 1975).

Received: July 7. 1978
Accepted: February 20, 1979

A. Girolami,
University of Padua Medical School,
Institute of Semeiotica Medica
Padua (Italy)

hemophilia B_M may have a prolonged Thrombotest, it is clear that the pattern has become complicated. Only by means of a battery of tests may a correct diagnosis be reached (table IV)

It is interesting to note that in several patients with factor VII deficiency a discrepancy between activity and CRM has already been demonstrated [4 6 9 23 24] These cases, which are gathered in table V suggest the existence of a great variability of the factor VII defect. On the basis of available data the following classification in four groups may be proposed (1) factor VII factor VII factor VII reduced, factor VII Padua. Other variants may also exist.

References

1 Alexander B., Goldstein, R., Landwehr G and Cook, C. D Congenital SPCA deficiency A hitherto unrecognized coagulation defect with hemorrhage rectified by serum and serum fractions. J clin. Invest. *30* 237–246 (1951).

2 Beck, E. A., Charace, P and Jackson, D P. A new inherited coagulation disorder caused by an abnormal fibrinogen (fibrinogen Baltimora) Nature, Lond. *208*. 143–145 (1965).

3 Bithell, Th. C., Pizarro, A., and McDiarmid, W D Variant of factor IX deficiency in female with 45-X Turner's syndrome. Blood *36* 169–176 (1970)

4 Briët, E., Loeliger E. A., Van Tilburg, N H., and Veltkamp, J J.. Molecular variants of factor VII. Thromb. Haemostasis *35* 289–294 (1976).

5 Denson, K. W., Biggs, R., and Mannucci, P M.. An investigation of three patients with Christmas disease due to an abnormal type of factor IX. J clin. Path. *21* 160–165 (1968)

6 Denson, K. W., Conrad, J and Samama, M Genetics variants of factor VII. Lancet *I*. 1234–1234 (1972).

7 Girolami, A Sticchi, A., Lazzarin, M., and Scarpa, R.. Congenital hypoprothrombinemia. Case report. Acta haemat. *44* 164–176 (1970).

8 Girolami, A., Molaro, G., Lazzarin, M., Scarpa, R., and Brunetti, A.. A new hemorrhagic condition due to the presence of an abnormal factor X (factor X Friuli). A study of a large kindred. Br J Haemat. *19* 179–192 (1970).

9 Girolami, A Venturelli, R., Righini, F., and Scarpa, R.. Parahemophilia. A case report. Acta haemat. *46* 242–253 (1971).

10 Girolami, A., Sticchi, A., and Brunetti, A.. Prothrombin level and activity on the abnormal factor X (factor X Friuli) hemorrhagic disorder Thromb. Diath. haemorrh. *25*. 147–155 (1971).

11 Girolami, A Lazzarin, M., Scarpa, R., and Brunetti, A.. Further studies on the abnormal factor X (factor X Friuli) coagulation disorder A report of another family Blood *37* 534–541 (1971).

12 Girolami, A., Cattarozzi, G., Mengarda, G and Lazzarin, M.. Congenital factor VII deficiency A case report. Blut *27* 236–242 (1973).

13 Girolami, A Scorza, P., Brunetti, A Morgagni, C., and Santini, G Congenital hypoproconvertinemia (factor VII deficiency). A report of two cases belonging to two different kindreds. Acta haemat. *50*· 228–237 (1973).

14 Girolami, A., Bareggi, G Brunetti, A., and Sticchi, A.. Prothrombin Padua. A 'new' congenital dysprothrombinemia. J Lab clin. Med. *84* 654–666 (1974).

15 Girolami, A., Brunetti, A Patrassi, G M., and Cafiero, F.. The K-Test (trypsin clotting time) in coumarin treated patients and in congenital deficiencies and abnormalities of the prothrombin complex. Blut *31* 291–298 (1975)

16 Girolami, A., Venturelli, R., Cella, G., Virgolini, L., and Burul, A.. Combined hereditary deficiency of factor VII and VIII. A distinct coagulation disorder due to the lack of an autosomal gene controlling factor VII and factor VIII activation? Acta haemat. *55* 181–191 (1976)

17 Girolami, A.; Falezza, G., Patrassi, G M. Stenico, M., and Vettore, L.. Factor VII Verona. Double heterozygosis between an abnormal factor VII and factor VII heterozygous deficiency Blood *50* 603–610 (1977).

18 Girolami, A., Fabris, F Dal Bo Zanon, R., Ghiotto, G and Burul, A.. A factor VII Padua.

technique, in order to evaluate the alkaline phosphatase activity in newly formed neutrophils.

Materials and Methods

8 patients with recently diagnosed CML were studied, they were either untreated or had not received any therapy for at least 1 month. All the patients had chronic-phase disease, splenomegaly a leukocyte count above 50×10^9/l, a percentage of mature granulocytes above 50% and immature granulocytes in blood smears. Low alkaline phosphatase scores in the peripheral blood neutrophils and Ph chromosome were present in all cases (table I). No clinical evidence of infection was found in any patient at the time of the study

Bone marrow cells were obtained from CML patients by sternum puncture.

Liquid cultures were performed in siliconized tubes as previously described [3]. Each tube contained 1×10^6 bone marrow cells in 1 ml of alfa-medium (Eurobio) supplemented with 20% human serum AB and 15% granulocyte-conditioned medium, prepared according to the method of *Price et al* [14]. Cultures were incubated at 37 °C in humidified incubator flushed with 5% CO_2 in air and were harvested at intervals of time.

Cell growth was determined using Coulter Counter (model Z BI) and the percentages of neutrophil cells were evaluated on Giemsa-stained smears. Viability of these cultures was determined by trypan blue dye exclusion.

NAP was evaluated, according to *Kaplow* [9], on smears made from the original bone marrow aspirates and from cells cultured at intervals of time from the liquid cultures. The films were fixed for 30 sec at 20 °C in methanol-formaldehyde solution, washed, air-dried and stained for 10 min using naphthol AS-BI phosphate (Sigma) as substrate and fast red violet LB (Sigma) as diazonium salt. At least 500 consecutive neutrophils were scored from 0 to 4 on the basis of intensity of staining and results expressed as NAP score for 100 cells.

Chromosome karyotyping was performed according to the method of *Rowley* [16].

Bone marrow granulocyte CFC were counted according to *Iscove et al.* [8]. Shortly unseparated 2×10^5 nucleated bone marrow cells were plated in 0.3% agar medium (alfa-medium) containing 15% of granulocyte-conditioned medium to stimulate activity. After 10 days of incubation, the colonies (> 40 cells) were counted.

Results

Neutrophil counts and NAP activity as a function of time in culture are shown in figure 1. Viable neutrophil counts increased to

Table I. Clinical and hematological data on CML patients

Patient	Age	Sex	HCT %	WBC 10^9/l	Platelets 10^9/l	Previous therapy	Karyotype	*In vitro* CFC/2 10^{5a}	NAP score[b]
M.C.	37	F	32	120	200	HU	Ph¹+	19	0
M.G.	50	M	38	180	400	HU	Ph¹+	64	3
D.G.	45	M	43	60	350		Ph. +	115	7
P.L.	35	F	37	430	420	–	Ph¹+	47	3
D.L.	72	F	36	80	340		Ph¹+	69	6
N.M.	40	F	42	150	400	–	Ph¹+	N.D	0
G.V.	36	M	42	170	100		Ph¹+	82	2
V.B.	50	F	34	170	500	–	Ph¹+	N.D	1

HU = Hydroxyurea. ND = not done.
Normal range 20–50.
Normal range 20–100.

Acta haemat. 62 12–16 (1979)

Alkaline Phosphatase Activity in Neutrophils of Chronic Myelocytic Leukemia Grown in Liquid Culture

F. Dallegri, M. Sessarego, R. Ghio and F. Patrone

Istituto Scientifico di Medicina Interna, Università di Genova, Genova

Key Words. Chronic myelocytic leukemia · Neutrophil alkaline phosphatase · Liquid culture

Abstract. Bone marrow cells from 8 Ph[1]-positive patients with chronic myelocytic leukemia (CML) have been cultured *in vitro*. Cellular growth in the presence of the Ph[1] chromosome and neutrophil alkaline phosphatase (NAP) have been evaluated at intervals of time during 19 days of culture. The mean viable neutrophil count reached a peak level on day 8, and the Ph[1] chromosome was present till day 10 suggesting an active proliferation of leukemic stem cells. NAP activity reached a maximun level in the same number of days as the neutrophil peak.

Our data suggest that newly formed neutrophils (Ph[1]+) in CML are normal as far as NAP activity is concerned. Moreover, they emphasize the hypothesis that in CML there is an accumulation of aged NAP negative cells, diluting positive neutrophils with a consequent low NAP score.

Decreased neutrophil alkaline phosphatase (NAP) activity is a well known feature of untreated patients with chronic myelocytic leukemia (CML) and it is currently used in the differentiation of CML from other myeloproliferative disorders [11, 12]. Nevertheless, the mechanism of this low enzyme activity is unknown. Some hypotheses have been suggested, such as absence of NAP synthesis directly related to the presence of the Ph[1] chromosome [1] and abnormal polymorph cytoplasmic maturation [13], accumulation of aged phosphatase-negative circulating neutrophils which might dilute the younger cells bearing enzymatic activity [7].

In 1973 *Chikkappa et al.* [2] cultured CML bone marrow cells in diffusion chambers implanted into the abdominal cavity of irradiated mice and observed a growth of highly NAP positive cells, suggesting an activation of the factors controlling the synthesis of the enzyme.

In order to avoid the influence of diffusible factors present in irradiated mice [5] bearing diffusion chambers, we grew CML bone marrow cells using a liquid culture

technique, in order to evaluate the alkaline phosphatase activity in newly formed neutrophils.

Materials and Methods

8 patients with recently diagnosed CML were studied: they were either untreated or had not received any therapy for at least 1 month. All the patients had chronic-phase disease, splenomegaly, leukocyte count above 50 × 10^9/l, a percentage of mature granulocytes above 50% and immature granulocytes in blood smears. Low alkaline phosphatase scores in the peripheral blood neutrophils and Ph chromosome were present in all cases (table I). No clinical evidence of infection was found in any patient at the time of the study.

Bone marrow cells were obtained from CML patients by sternum puncture.

Liquid cultures were performed in siliconized tubes as previously described [3]. Each tube contained 1 × 10^6 bone marrow cells in 1 ml of alfa-medium (Eurobio) supplemented with 20% human serum AB and 15% granulocyte-conditioned medium, prepared according to the method of *Price et al.* [14]. Cultures were incubated at 37 °C in humidified incubator flushed with 5% CO_2 in air and were harvested at intervals of time.

Cell growth was determined using Coulter Counter (model Z BI) and the percentages of neutrophil cells were evaluated on Giemsa-stained smears. Viability of these cultures was determined by trypan blue dye exclusion.

NAP was evaluated, according to *Kaplow* [9], on smears made from the original bone marrow aspirates and from cells cultured at intervals of time from the liquid cultures. The films were fixed for 30 sec at 20 °C in methanol-formaldehyde solution, washed, air-dried and stained for 10 min using naphthol AS-BI phosphate (Sigma) as substrate and fast red violet LB (Sigma) as diazonium salt. At least 500 consecutive neutrophils were scored from 0 to 4 on the basis of intensity of staining and results expressed as NAP score for 100 cells.

Chromosome karyotyping was performed according to the method of *Rowley* [16].

Bone marrow granulocyte CFC were counted according to *Iscove et al.* [8]. Shortly, unseparated 2 × 10^5 nucleated bone marrow cells were plated in 0.3% agar medium (alfa-medium) containing 15% of granulocyte-conditioned medium to stimulate activity. After 10 days of incubation, the colonies (> 40 cells) were counted.

Results

Neutrophil counts and NAP activity as a function of time in culture are shown in figure 1. Viable neutrophil counts increased to

Table I. Clinical and hematological data on CML patients

Patient	Age	Sex	HCT %	WBC 10^9/l	Platelets 10^9/l	Previous therapy	Karyotype	*In vitro* CFC/2 10^{5a}	NAP score[b]
M.C.	37	F	32	120	200	HU	Ph¹+	19	0
M.G.	50	M	33	180	400	HU	Ph¹+	64	3
D.G.	45	M	43	60	350	–	Ph¹+	115	7
P.L.	35	F	37	430	420		Ph +	47	3
D.L.	72	F	36	80	340		Ph +	69	6
N.M.	40	F	42	150	400	–	Ph +	N.D	0
G.V.	36	M	42	170	100		Ph +	82	2
V.B.	50	F	34	170	500	–	Ph +	N.D	1

HU = Hydroxyurea. ND = not done.
Normal range: 20–50.
Normal range: 20–100.

Acta haemat. 62 12–16 (1979)

Alkaline Phosphatase Activity in Neutrophils of Chronic Myelocytic Leukemia Grown in Liquid Culture

F Dallegri M Sessarego R Ghio and F Patrone

Istituto Scientifico di Medicina Interna, Università di Genova, Genova

Key Words. Chronic myelocytic leukemia Neutrophil alkaline phosphatase Liquid culture

Abstract. Bone marrow cells from 8 Ph[1] positive patients with chronic myelocytic leukemia (CML) have been cultured *in vitro* Cellular growth in the presence of the Ph[1] chromosome and neutrophil alkaline phosphatase (NAP) have been evaluated at intervals of time during 19 days of culture The mean viable neutrophil count reached a peak level on day 8 and the Ph[1] chromosome was present till day 10 suggesting an active proliferation of leukemic stem cells NAP activity reached a maximum level in the same number of days as the neutrophil peak.

Our data suggest that newly formed neutrophils (Ph[1]+) in CML are normal as far as NAP activity is concerned. Moreover they emphasize the hypothesis that in CML there is an accumulation of aged NAP-negative cells, diluting positive neutrophils with a consequent low NAP score

Decreased neutrophil alkaline phosphatase (NAP) activity is a well known feature of untreated patients with chronic myelocytic leukemia (CML) and it is currently used in the differentiation of CML from other myeloproliferative disorders [11 12] Nevertheless, the mechanism of this low enzyme activity is unknown. Some hypotheses have been suggested, such as absence of NAP synthesis directly related to the presence of the Ph[1] chromosome [1] and abnormal polymorph cytoplasmic maturation [13] accumulation of aged phosphatase-negative circulating neutrophils which might dilute the younger cells bearing enzymatic activity [7]

In 1973 *Chikkappa et al* [2] cultured CML bone marrow cells in diffusion chambers implanted into the abdominal cavity of irradiated mice and observed a growth of highly NAP-positive cells, suggesting an activation of the factors controlling the synthesis of the enzyme.

In order to avoid the influence of diffusible factors present in irradiated mice [5] bearing diffusion chambers, we grew CML bone marrow cells using a liquid culture

formed granulocytes without the interference of storage compartments, we exclude that the extremely low enzymatic activity in CML is entirely due to an intrinsic cellular defect, involving chromosomal aberration and defective mechanism for transfer of the genetic message as suggested by *Chikkappa et al.* [2]

This explanation is supported also by the observation that in CML patients high NAP scores may be found during phases of depletion of the granulocyte pool with active bone marrow regeneration [6, 15]

We conclude that in the chronic phase of CML, besides an increased myelopoietic capacity with the potential for normal cell maturation, as demonstrated by *Golde et al.* [4] normal mechanisms of NAP synthesis are also present.

References

1 Alter A. A.; Dobkin, G., Pourfar M., Rosner, F and Lee, S. L. Genes on the mongol chromosome. Lancet *i.* 506 (1963).

2 Chikkappa, G., Boecker, A. R., Borner G., Carsten, A. L., Conkling, K.; Cook, L., Cronkite, E. P and Burwoody S. Return of alkaline phosphatase in chronic myelocytic leukaemia cells in diffusion chamber cultures. Proc. Soc. exp. Biol. Med. *143.* 212–217 (1973).

3 Ghio, R., Bianchi, G., Löwenberg, B., Dicke, K. A., and Ajmar F.: Effects of fibroblasts on the growth of erythroid progenitor cells *in vitro.* Expl Hemat. *5* 341–347 (1977).

4 Golde, D. W Byers, L. A., and Cline, M. S. Chronic myelogenous leukemia cell growth and maturation in liquid culture. Cancer Res *34* 419–423 (1974).

5 Greenberg, T S., Hassan, L. R. Karpas, A., France, D. S., and Moloney W C.: Leucocyte alkaline phosphatase elevation in human leukaemia-derived cell-lines cultured in diffusion chambers. Scand. J Haematol. *19* 242–254 (1977).

6 Hayhoe, F G J and Quaglino, D.. Cytochemical demonstration and measurement of leucocyte alkaline phosphatase activity in normal and pathological states by a modified azo-dye coupling technique. Br J Haemat. *4* 375–389 (1958).

7 Hook, E. B. and Engel, R. R.. Leucocyte life-span, leucocyte alkaline phosphatase, and the 21st chromosome. Lancet *i.* 112 (1964).

8 Iscove, N. N Senn, J S., Till, E., and McCulloch, E. A.. Colony formation by normal and leukemic human marrow cells in culture: effect of conditioned medium from human leukocytes. Blood *37* 1 15 (1971).

9 Kaplow L. S.. Cytochemistry of leucocyte alkaline phosphatase. Use of complex naphthol AS phosphate in azo-dye coupling technics Am. J clin. Path. *39* 439–449 (1963).

10 Kelemen, E.. Granulocyte alkaline phosphatase activity measure of the emergence time of mature marrow neutrophils. Acta haemat. *50* 19– 4 (1973).

11 Kober R. D Seaman, A. J Osgood, E. E., and Van Bellinghen, P Myeloproliferative disease: diagnostic value of the leukocyte alkaline phosphatase test. Am. J clin. Path. *30* 295–301 (1958).

12 Mitus, W J Bergna, L. J Mednicoff, I. B., and Dameshek, W Alkaline phosphatase of mature neutrophils in chronic forms of the myeloproliferative syndrome. Am. J clin. Path. *30* 285–294 (1958).

13 Pedersen, B. and Hayhoe, F G. J Cellular changes in chronic myeloid leukaemia. Br. J Haemat. *21* 251-256 (1971).

14 Price, G. B., McCulloch, E. A., and Till, J E A new human low molecular weight granulocyte stimulating activity Blood 42. 341 348 (1973).

15 Rosner F Schreiber, Z. R., and Parise, F Leucocyte alkaline phosphatase. Fluctuation with disease status in chronic granulocytic leukaemia. Archs intern. Med. *130* 892–894 (1972).

16 Rowley J D.. A new consistent chromosomal abnormality in chronic myelogenous leukaemia identified by quinacrine fluorescence and Giemsa staining. Nature, Lond. *243* 290–293 (1973).

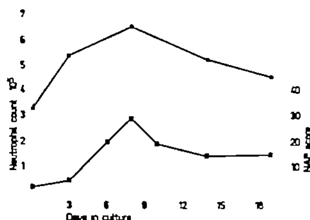


Fig. 1. Variation of neutrophils count (●) and NAP scores (■) during culture days.

reach the maximum level on day 8, showing a nearly 100% rise. The Ph[1] chromosome was present in 60–80% of the metaphases examined in the original bone marrow and in 100% of the mitoses harvested from the cultured cells. On day 10 a moderate number of mitoses was still present, all these mitoses were Ph[1]-positive.

NAP score reached the peak on day 8 and a decrease of the NAP negative neutrophils was noted. The percentage of the neutrophils lacking enzyme activity was 98% in the original bone marrow specimens, 95% on day 3 89% on day 6 77% on day 8, 88% on day 10 93% on day 14 and 92% on day 19 Cells bearing maximum alkaline phosphatase activity (grade 4) were uncommon also on day 8.

Bone marrow CFC, evaluated in 6 of our patients, were increased in 4 cases in comparison with normal controls (table I)

Discussion

The increase in viable neutrophils in culture and the presence of the Ph[1] chromosome in all evaluable mitoses provide strong evidence that the *in vitro*-developed mature granulocytes derive from a Ph[1]-positive stem cell and are all virtually carrying this chromosome marker

On day-8 culture the neutrophils show normal alkaline phosphatase activity and the positive cells increase in comparison with the original bone marrow samples. It, therefore, appears that neutrophils placed in culture progressively die by senescence and are replaced by a population of younger cells bearing normal enzyme activity In fact, it has been demonstrated that the alkaline phosphatase level of a neutrophil is inversely related to its age [18]

This is also in agreement with our preliminary studies of bone marrow from normal subjects and from patients with non-hematological diseases in whom we have found an increase in NAP activity in liquid culture roughly proportional to myeloid proliferation [unpubl.] In bone marrow from normal subjects as well as from CML patients, the NAP score is lower than in peripheral blood. This may be due to the dilution of young mature NAP-positive neutrophils by band forms other than by older cells since the marrow is also a storage compartment. In fact in cases of depletion of bone marrow granulocyte reserve, an increased NAP activity has been found in bone marrow as well as in peripheral blood of non-CML patients [10]

Our *in vitro* data seem to fit well the hypothesis of *Spiers et al.* [17] that in CML newly formed neutrophils normal in respect to the phosphatase content are rapidly sequestered in non-circulating pools such as spleen, liver bone marrow itself with a consequent low NAP score. From the rise in NAP activity in our cultures, where we can examine the entire population of newly

Acta haemat. 62: 17–19 (1979)

Bone Marrow Morphology in Acute Eosinophilic Monocytic Leukaemia

U. Sjögren

Departments of Clinical Chemistry and Internal Medicine, University Hospital, Lund

Key Words. Bone marrow · Eosinophils · Leukaemia · Mitotic index · Monocytes

Abstract. The mitotic indices of the granulopoietic precursor cells in the bone marrow of 3 untreated patients with acute eosinophilic monocytic leukaemia were found to be significantly lower than those of 20 normals. The differences were found in the eosinophilic, the neutrophilic and the monocytic precursors indicating leukaemic involvement with a concomitant maturation arrest in all the three cell lines.

The separation of the acute myeloid leukaemias in several cytologic subgroups has lead to quite different therapeutic regimes, sometimes with promising results, e.g. in the acute promyelocytic leukaemia and the acute monocytic leukaemia. Leukaemic involvement of the eosinophilic cell line has ever been questioned but low mitotic indices of the eosinophilic precursor cells in combination with dominance of morphologically abnormal eosinophils seem to justify the diagnosis of eosinophilic leukaemia [5, 6]. The eosinophilic-monocytic leukaemia is a rare neoplastic condition with myeloblasts and immature monocytes dominating in the peripheral blood but with a marked increase of atypical immature eosinophils in the bone marrow [2, 4, 8]. It has been suggested that the bone marrow eosinophilia found in some cases of acute myeloid leukaemias is a reactive one [for review, see ref. 1, 5] and the aim of the present work was to find out if the eosinophils and the neutrophils were affected by the leukaemic process.

Materials and Methods

Patients

3 patients and 20 haematologically healthy persons were included in this study. Pertinent bone marrow data are given in table I.

The first patient, B. J., 25-year-old man, was admitted on December 12th, 1968. Hb was 10.2 g/dl, WBC 29.1 × 10^9/l with 74.5% monocytes and promonocytes, 6% myeloblasts and only 1% eosinophils. S-lysozyme 80 mg/l and U-lysozyme 780 mg/l. He was treated with VAMP (vincristine, methylaminopterine, 6-mercaptopurine and prednisolone) and got 3 remissions. The patient died after 20 months and the autopsy showed hepatosplenomegaly, haemorrhagic diathesis and widely spread leukaemic infiltrates.

17 Spiers, A. S. D. Liew, A., and Baikie, A. G.: Neutrophil alkaline phosphatase score in chronic granulocytic leukaemia: effects of splenectomy and antileukaemic drugs. J. clin. Path. *28*: 517–523 (1975).
18 Williams, D. M.: Leukocyte alkaline phosphatase as a marker of cell maturity: a quantitative cytochemical and autoradiographic study. Br. J. Haemat. *31*: 371–379 (1975).

Received: July 7, 1978
Accepted: December 16, 1978

Dr. Franco Dallegri,
Istituto Scientifico di Medicina Interna,
Università
Viale Benedetto XV
16132 Genova (Italy)

In the leukaemic patients 14–49% of the neutrophilic cells in the bone marrow were neutrophilic promyelocytes compared to 2–8% (median 5) in the normals. The difference is significant ($p < 0.002$).

Discussion

It is not unusual to find eosinophils with morphologic changes in various types of myeloid leukaemias. Thus abnormally large eosinophilic granules, mixed basoeosinophilic granules, nuclear-cytoplasmatic asynchrony and vacuoles in the cytoplasm have been recorded [2, 4, 5, 8] but morphology alone is insufficient to prove that these changes do not occur in diseases with a reactive eosinophilia. A significant 'shift to the left' within the eosinophilic bone marrow pool with an accumulation of early precursors, especially promyelocytes, has also been found in eosinophilic leukaemias [1, 3, 5, 7] and this is verified in the present report.

It has earlier been shown that determinations of the mitotic indices of the granulopoietic precursor cells in the bone marrow might be of diagnostic and prognostic significance in various types of myeloid leukaemias including the eosinophilic ones [5, 6]. Although the mitotic countings are seriously affected by the admixture of peripheral blood low mitotic indices of the precursors may indicate leukaemic involvement of these cells [5, 6]. In the present investigation there was a significant decrease in the mitotic activity of the monocytic, the eosinophilic and the neutrophilic precursors concomitant with a maturation arrest in all the three cell lines. The acute monocytic-eosinophilic leukaemia seems to be a variant of the acute myelomonocytic leukaemia with engagement of all granulopoietic cell lines.

References

1 Brandt, L., Mitelman, F., Beckman, G., Lanell, H. and Nordenson, I.: Different composition of the eosinophilic bone marrow pool in reactive eosinophilia and eosinophilic leukaemia. Acta med. scand. *201:* 177–180 (1977).

2 Leder, L. D.: Akute myelo-monocytäre Leukämie mit atypischen Naphthol-AS-D-Chloracetat-Esterase-positiven Eosinophilen. Acta haemat. *44:* 52–62 (1970).

3 Liso, V., Troccoli, G., Specchia, G., and Magno, M.: Cytochemical 'normal' and 'abnormal' eosinophils in acute leukemias. Am. J. Hematol. 2: 123–131 (1977).

4 Ruzicka, P., Pawlowsky, J., Erber, A. und Nowotny, H.: Drei Fälle von Eosinophilenleukämie mit atypischer Granulation in Eosinophilen und Neutrophilen. Blut *32:* 337–346 (1976).

5 Sjögren, U.: Low mitotic activity in eosinophilic leukaemia. Acta haemat. *55:* 153–159 (1976).

6 Sjögren, U.: Mitotic activity in myeloid leukaemias. Scand. J. Haematol. *20:* 159–167 (1978).

7 Stavem, P., Ly, B., Bilchfeldt, P. and Andreassen, P.: Acute eosinophilo-myelomonocytic leukaemia, one of the 'in between leukaemias'. Scand. J. Haematol. *21:* 355–359 (1978).

8 Wulfhekel, U. und Dullmann, J.: Zur Ultrastruktur der eosinophil-myelomonocytären Leukämie; in Stacher und Höcker: Erkrankungen der Myelopoese, pp. 413–416 (Urban & Schwarzenberg, Berlin 1976).

Received: August 16, 1978
Accepted: December 8, 1978

U. Sjögren, Departments of Clinical Chemistry and Internal Medicine, University Hospital, Lund (Sweden)

The second patient, S. A. S., a 42 year-old man, was admitted on June 9th, 1970 Hb was 7.5 g/dl WBC 47.4 × 10⁹/l with 56.5% monocytes and promonocytes, 13% myeloblasts and 2% eosinophils. Treatment with VAMP was started but the patient rapidly got severe metabolic acidosis and died within 4 days. Autopsy was not performed.

The third patient, M. K., a 27-year-old woman, was admitted on April 13th, 1978. Hb was 5.3 g/dl, WBC 62.0 × 10⁹/l with 45% monocytes and promonocytes, 19% myeloblasts and 2% eosinophils. Treatment with cytosine arabinoside and rubidomucin was not successful and this patient died on April 28th, 1978. The autopsy revealed hepatosplenomegaly haemorrhagic diathesis and multiple leukaemic infiltrates.

Normals

11 women and 9 men, 19–82 years old, without perceivable haematologic disorders, and with clinical diagnoses such as adipositas, cervical disc prolapse, spondylolisthesis, psychiatric disorders and unverified hypogonadismus, served as normals. Hb 12.1–16.6 g/dl, WBC 4.2–8.3 × 10⁹/l and ESR 2–23 mm.

Bone Marrow Examination

May-Grünwald-Giemsa stained bone marrow smears were investigated at the time of diagnosis. A differential count of 1,000 nucleated cells was performed and the cells were classified according to *Sjögren* [5, 6]. Mitotic indices of the granulopoietic precursor cells were calculated through examination of 5 730–11,970 such cells. At least 36 mitoses were counted in each patient.

Statistics

Non-parametric statistics were used. The Mann Whitney two-tailed U test corrected for ties was used to assert significance of the results.

Results

The mitotic indices of the granulopoietic precursors in the leukaemia patients were significantly lower than those of the normals ($p < 0.002$ cf table II)

In the leukaemia group 12–49% of the bone marrow eosinophils were promyelocytes compared to 3–12% (median 6) in the normals The difference is significant ($p < 0.02$)

72–92% of the monocytic cells were classified as promonocytes in the leukaemia patients compared to 21–48% (median 35) in the normals The difference is significant ($p < 0.002$)

Table I. Bone marrow data from 3 patients with acute eosinophilic-monocytic leukaemia (values give percentage of all nucleated cells)

Cell line	Patient B. J	Patient S. A. S.	Patient M. K
Monocytic	46.4	24.3	47.4
Eosinophilic	35.8	25.4	17.1
Neutrophilic	4.2	16.1	7.8
Myeloblasts	6.3	17.6	17.6
Erythroblasts	2.0	8.5	2.0

Table II. Mitotic data of 3 patients with acute eosinophilic-monocytic leukaemia and of 20 normals

Cell line	Patients B J	Patients S. A. S.	Patients M K	Normals (median values)
Monocytic	1.1	0.65	0.28	1.7
Eosinophilic	0.54	0.61	0.44	1.3
Neutrophilic	–	0.73	0.64	1.1
Myeloblasts	–	0.64	0.28	2.4
All precursors	0.92	0.66	0.33	1.[illegible]

The separate indices of the normal granulopoietic precursor cells have been estimated by *Sjögren* [5, 6]. Figures give percentage of mitoses in 'mitotable' cells, e.g., myeloblasts, promyelocytes, myelocytes and promonocytes.

day 0. *Lymphocyt cytotoxicity tests* and *histocompatibility testing* were done by the standard microlymphocyte cytotoxicity method [8] and mixed leucocyte culture tests [6]. *Cytogenetic studies* were performed by two methods: (1) determination of the sex chromosome in PHA-stimulated peripheral lymphocytes [1], and (2) the quinacrine-mustard technique for identification of the Y body in interphase nuclei on bone marrow smears [9]. Control of interphase uclei of the marrow donor demonstrated strong fluorescence of his Y chromosome. For *plasma exche ge* the Aminco blood cell separator was used [5]. The plasma removed from the patient was replaced volume-to-volume by fresh plasma of normal male donors of blood group AB. *Routine laboratory methods* were used for blood chemistry hematologic examinations, Coombs and Ham test, determination of anti-DNA antibodies as well as red cell serology.

Table I. Peripheral blood counts prior to and after bone marrow transplantation attempt

	3-3-76	9-20-76	2-16-77	9-5-78
Hematocrit, %	35	13	25	42
Reticulocytes/mm³	70,000	55,000	0	153,000
Granulocytes/mm³	800	450	185	2,800
Platelets/mm³	81,000	13,000	5,000	95,000

Value following red cells transfusions.

Case Report

A 17-year-old hite female hairdresser was in good health until March 1976, hen she came for medical attention due to a left-sided renal calculus and cystopyelitis. Mild pancytopenia was noted (table I). She rapidly recovered from infection and did well until September 20, 1976, when she was first admitted to our hospital with pyrexia. Clinical examination revealed pallor dyspnea, pedal edema and cystopyelitis. Hematologic data were consistent with aplastic anemia (table I) bone ma row aspiration and biopsy demonstrated pronounced hypocellularity with 60% erythroid and 20% myeloid elements, 10% lymphocytes and no megacaryocytes. All other routine laboratory investigations including serum vitamin B and folic acid levels revealed normal values. Direct Coombs test, Ham test and anti-DNA antibodies were negative. There was history of exposure to several potentially causative drugs including oxy

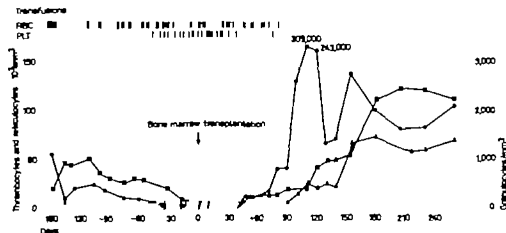


Fig. 1. Course of peripheral blood cell counts prior to and after bone marrow transplantation. ● = Reticulocytes; ■ = granulocytes; ▼ = thrombocytes, counts prior to substitutions.

Acta haemat. 62 20–24 (1979)

Autologous Hematologic Recovery from Aplastic Anemia following High Dose Cyclophosphamide and HLA-Matched Allogeneic Bone Marrow Transplantation

J Gmür A von Felten K Rhyner and P G Frick

Department of Internal Medicine, University Hospital of Zürich, Zürich

Key Words. Aplastic anemia Autologous bone marrow recovery Bone marrow transplantation

Abstract. A 17 year-old woman with severe aplastic anemia was treated with high-dose cyclophosphamide followed by infusion of bone marrow cells from her HLA identical ABO-incompatible brother The marrow graft failed to take Subsequently the patient revealed an autologous marrow reconstitution leading to a near-complete hematologic recovery which is now persisting for over 20 months.

Introduction

Since severe aplastic anemia still remains a disorder of poor prognosis in spite of remarkable advances in supportive care [3 7] bone marrow transplantation has been introduced as a new therapeutic approach in such patients [15] Provided that HLA identical siblings are used as marrow donors, promising results may be achieved [12] Intensive immunosuppression of the recipient with chemotherapeutic agents or total body irradiation prior to transplantation seem indispensable in order to permit engraftment of donor marrow cells. However failure of engraftment or graft rejection occur in 25–40% of transplanted recipients which are usually associated with a rapidly fatal course due to complete and permanent marrow aplasia [4 12]

Recently 4 patients given high dose cyclophosphamide followed by bone marrow transplantation from an HLA matched sibling who rejected the marrow graft but subsequently demonstrated an unexpected recovery of their own marrow function have been reported [10 11 13 16] Since the biological mechanism operating in these patients remains unclear the report of an additional case differing from all previously published patients by an unusually long interval between diagnosis of aplastic anemia and bone marrow transplantation attempt may be of interest.

Material and Methods

Bone marrow transplantation was performed according to the protocol of the Seattle group [14]. The day of transplantation is designated as

day 0. *Lymphocyte cytotoxicity tests* and *histocompatibility testing* were done by the standard microlymphocyte cytotoxicity method [8] and mixed leucocyte culture tests [6]. *Cytogenetic studies* were performed by two methods: (1) determination of the sex chromosome in PHA-stimulated peripheral lymphocytes [1], and (2) the quinacrine-mustard technique for identification of the Y body in interphase nuclei on bone marrow smears [9]. Control of interphase nuclei of the marrow donor demonstrated strong fluorescence of his Y chromosome. For *plasma change* the Aminco blood cell separator was used [5]. The plasma removed from the patient was replaced volume-to-volume by fresh plasma of normal male donors of blood group AB. *Routine laboratory methods* were used for blood chemistry, hematologic examinations, Coombs and Ham test, determination of anti-DNA antibodies as well as red cell serology.

Table I. Peripheral blood counts prior to and after bone marrow transplantation attempt

	3-3-76	9-20-76	2-16-77	9-5-78
Hematocrit, %	35	13	25	4_
Reticulocytes/mm³	70,000	55,000	0	153,000
Granulocytes/mm³	800	450	185	2,800
Platelets/mm³	81,000	13,000	5,000	95,000

Value following red cells transfusions.

Case Report

A 17-year-old white female hairdresser was in good health until March 1976, when sh came for medical ttention due to left-sided renal calculus and cystopyelitis. Mild pancytopenia was noted (tabl I). She rapidly recovered from infection and did well until September 20, 1976, when she was first admitted to our hospital with pyrexia. Clinical examination revealed pallor dyspnea, pedal edema and cystopyelitis. Hematologic data were consistent with aplastic anemia (table I): bone marrow aspiration and biopsy demonstrated pronounced hypocellularity with 60° erythroid and 20°/ myeloid elements, 10° lymphocytes and no megacaryocytes. All other routine laboratory investigations including serum vitamin B and folic acid levels revealed normal values. Direct Coombs test, Ham test and anti-DNA antibodies were negative. There was history of exposure to several potentially causative drugs including oxy

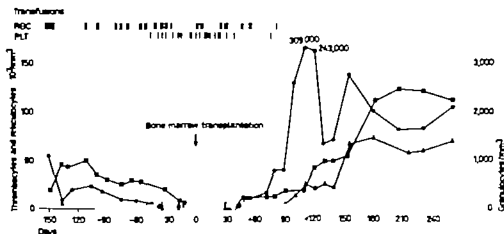


Fig. 1. Course of peripheral blood cell counts prior to and after bone marrow transplantation. ● = Reticulocytes; ■ = granulocytes, ▼ = thrombocytes, counts prior to substitutions.

phenbutazone. During the following 5 months a progressive drop of peripheral blood counts (fig. 1) and bone marrow cellularity ensued and the clinical course was complicated by repeated episodes of infection and hemorrhage. In addition, multispecific HLA antibodies became detectable in lymphocyte cytotoxicity testing. Up to February 1977 she needed a total of 61 red cell transfusions and 106 platelet concentrates.

Since histocompatibility testing revealed an HLA-identical, MLC nonreactive but ABO-incompatible brother (donor blood group B recipient A) bone marrow transplantation was attempted on February 21 1977 The patient was immunosuppressed with 50 mg of cyclophosphamide/kg body weight on each of 4 consecutive days followed 36 h later by the infusion of 2.1 × 10⁸ nucleated marrow cells/kg body weight. Plasma exchanges performed immediately before bone marrow infusion (22 liters) and on day +6 after transplantation (14 liters) reduced the patient's antibodies directed against the red cell antigen B of the donor After transplantation, methotrexate was given in order to prevent graft vs. host disease. Bone marrow aspirates of days +10, +17 and +25 post transplantation revealed an aplastic marrow with only a few lymphoid cells. Those were of recipient type as evidence by quinacrine-mustard staining. On day +11 the patient experienced a left-sided cystopyelitis *(Escherichia coli)* with subsequent septicemia and soft tissue abscess formation. She recovered from this severe infection under multiple antibiotics and repeated granulocyte transfusion. Bone marrow aspirate of day +44 although still markedly hypocellular was repopulated by some islands of erythroid and myeloid elements again of recipient type. Around day +50 reticulocytes and granulocytes reappeared in the peripheral blood Over the following weeks, bone marrow and peripheral blood counts gradually improved and all transfusions could be discontinued (fig. 1) Bone marrow biopsy of day + 120 showed abundant erythropoiesis and megacaryocytes but still reduced myelopoiesis. Chromosome marker studies of marrow cells performed by quinacrine-mustard staining indicated that all these hemopoietic cells were of recipient's origin. In addition, peripheral red cells were uniformly of blood group A as before transplantation attempt and 30 out of 30 mitoses of peripheral lymphocytes were found to be female. No signs of graft vs. host disease in the postgrafting period occurred. The patient was discharged from the laminar air flow unit 120 days after transplantation. She regained a normal active life in good health without any medication or transfusions. Hemoglobin and white blood cells have remained within normal limits for the last 16 months, but a mild thrombocytopenia (65 000 to 135,000/mm³) persisted.

Discussion

Most patients with severe aplastic anemia immunosuppressed with high-dose cyclophosphamide followed by bone marrow transplantation from HLA-identical donors demonstrate prompt marrow repopulation and hematologic recovery with cells of donor type [4 12] However secondary graft rejection or failure of engraftment still occurs in 25–40% usually leading to profound marrow aplasia and death [12, 16] Recently 4 patients have been reported who demonstrated an unexpected autologous marrow recovery after rejection or nontake of an HLA matched marrow transplantation preceded by high-dose cyclophosphamide [10 11 13 16] One additional patient with severe aplastic anemia ran a similar course after high-dose cyclophosphamide alone [2] Since the interval between the first manifestations of aplastic anemia and bone marrow transplantation was rather short in these cases (3 weeks to 2 months) their unexpected course may have been a fortuitous spontaneous recovery It is well known, that such recoveries in severe aplastic anemia, if ever occur within a few weeks in most cases [3] Therefore, this long time lag of 11 months interposed between diagnosis and bone marrow transplantation attempt in our patient, together with the severity of the aplastic anemia in the last 5 months prior to

transplantation may be an argument against the hypothesis of spontaneous recovery Moreover *in contrast to 3 of the other 5 cases [2, 13 16] treatment with androgens* and/or steroids after transplantation was avoided in our patient, thus excluding re sponse to such a therapy

On the other hand, this unexpected course of events may be related in some way to the bone marrow transplantation manoeuvre, especially to the immunosuppressive action of high-dose cyclophos phamide. The observation of recovery from severe aplastic anemia after treatment with cyclophosphamide alone [2] is supporting the concept that in some patients aplastic anemia may have an autoimmune pathogen esis. But for the present time it remains unknown how frequent autologous marrow recovery could be expected after such a *transplantation procedure not resulting* in permanent engraftment. In all published cases including our own, definite signs of marrow reconstitution could be observed 40–60 days following high-dose cyclophosphamide at the earliest. However many patients with failing engraftment or early graft rejection succombed to infection and hemorrhage or were retransplanted clearly before autologous marrow recovery would have been expected to occur Thus, the incidence of autologous marrow recovery fol lowing high-dose cyclophosphamide and graft rejection cannot be assessed for the present time. Likewise, treatment of severe aplastic anemia with immunosuppressive agents alone still remains a highly experimental therapy Additional case reports of recovery from aplastic anemia after such treatments are needed to clarify the possible role of immunosuppression in selected patients.

References

1 *Arakaki, D T and Sparkes, R. S* Microtechnique for culturing leukocytes from whol blood. Cytogenetics 2 57–66 (1963)

2 Baran, D T Griner P F and Klemperer M. R.. Recovery from aplastic anemia after treatment with cyclophosphamide. New Engl. J Med *295* 1522 1523 (1976).

3 Camitta, B. M Rappaport, J M Parkman, R., and Nathan, D G Selection of patients for bone marrow transplantation in severe aplastic anemia. Blood *45* 355–363 (1975).

4 Gluckman, E Devergie, A Marty M Bussel, A., Rottembourg, J Dausset, J and Bernard, J Allogeneic bone marrow transplantation in aplastic anemia – report of 25 cases. Transplantn Proc. *10* 141–145 (1978)

5 Graw R. G Buckner C D and Eisel, R. J Plasma exchange transfusion for hepatic coma. Transfusion *10* 260–267 (1970).

6 Hartzmann, R. J Segall, M., Bach, M. L., and Bach, F H.. Miniaturization of the mixed leukocyte culture test A preliminary report Transplantation *11* 268–273 (1971).

7 Lohrmann, H. P Kern, P Niethammer D and Heimpel, H Identification of high-risk patients with aplastic anemia in selection for allogeneic bone-marrow transplantation. Lancet *I* 647–550 (1976).

8 Mittal, K. K., Mickey M R Singal, D P and Terasaki, P I Serotyping for homotransplantation. Refinement of microdroplet lymphocyte cytotoxicity test. Transplantation *6* 913–927 (1968).

9 Pearson, P L Bobrow C D and Vosa, C G.. Technique for identifying Y chromosomes in human interphase nuclei. Nature, Lond. *226*. 78–82 (1970)

10 *Sensenbrenner L. L., Steele, A A., and Santos*, G W Recovery of hematologic competence without engraftment following attempted bone marrow transplantation for aplastic anemia. Expl Hematol. *5* 51 58 (1977).

11 Speck, B., Cornu, P Jeannet, M., Nissen, C Burri, H. P Groff, P Nagel, G A., and Buckner C. D Autologous marrow recovery following allogeneic marrow transplantation in patient with severe aplastic anemia. Expl Hematol. *4* 131 136 (1976).

phenbutazone. During the following 5 months a progressive drop of peripheral blood counts (fig. 1) and bone marrow cellularity ensued and the clinical course was complicated by repeated episodes of infection and hemorrhage. In addition, multispecific HLA antibodies became detectable in lymphocyte cytotoxicity testing. Up to February 1977 she needed a total of 61 red cell transfusions and 106 platelet concentrates.

Since histocompatibility testing revealed an HLA identical, MLC nonreactive but ABO-incompatible brother (donor blood group B, recipient A) bone marrow transplantation was attempted on February 21, 1977. The patient was immunosuppressed with 50 mg of cyclophosphamide/kg body weight on each of 4 consecutive days followed 36 h later by the infusion of 2.1 × 10⁸ nucleated marrow cells/kg body weight. Plasma exchanges performed immediately before bone marrow infusion (22 liters) and on day +6 after transplantation (14 liters) reduced the patient's antibodies directed against the red cell antigen B of the donor. After transplantation, methotrexate was given in order to prevent graft vs. host disease. Bone marrow aspirates of days +10, +17 and +25 posttransplantation revealed an aplastic marrow with only a few lymphoid cells. Those were of recipient type as evidence by quinacrine-mustard staining. On day +11, the patient experienced a left-sided cystopyelitis *(Escherichia coli)* with subsequent septicemia and soft tissue abscess formation. She recovered from this severe infection under multiple antibiotics and repeated granulocyte transfusion. Bone marrow aspirate of day +44 although still markedly hypocellular was repopulated by some islands of erythroid and myeloid elements again of recipient type. Around day +50 reticulocytes and granulocytes reappeared in the peripheral blood. Over the following weeks, bone marrow and peripheral blood counts gradually improved and all transfusions could be discontinued (fig. 1). Bone marrow biopsy of day + 120 showed abundant erythropoiesis and megacaryocytes but still reduced myelopoiesis. Chromosome marker studies of marrow cell performed by quinacrine-mustard staining indicated that all these hemopoietic cells were of recipient's origin. In addition, peripheral red cells were uniformly of blood group A as before transplantation attempt and 30 out of 30 mitoses of peripheral lymphocytes were found to be female. No signs of graft vs. host disease in the postgrafting period occurred. The patient was discharged from the laminar air flow unit 120 days after transplantation. She regained a normal active life in good health without any medication or transfusions. Hemoglobin and white blood cells have remained within normal limits for the last 16 months, but a mild thrombocytopenia (65 000 to 135 000/mm³) persisted.

Discussion

Most patients with severe aplastic anemia immunosuppressed with high-dose cyclophosphamide followed by bone marrow transplantation from HLA identical donors demonstrate prompt marrow repopulation and hematologic recovery with cells of donor type [4, 12]. However secondary graft rejection or failure of engraftment still occurs in 25–40%, usually leading to profound marrow aplasia and death [12, 16]. Recently 4 patients have been reported who demonstrated an unexpected autologous marrow recovery after rejection or nontake of an HLA matched marrow transplantation preceded by high-dose cyclophosphamide [10, 11, 13, 16]. One additional patient with severe aplastic anemia ran a similar course after high-dose cyclophosphamide alone [2]. Since the interval between the first manifestations of aplastic anemia and bone marrow transplantation was rather short in these cases (3 weeks to 2 months) their unexpected course may have been a fortuitous spontaneous recovery. It is well known that such recoveries in severe aplastic anemia if ever occur within a few weeks in most cases [3]. Therefore, this long time lag of 11 months interposed between diagnosis and bone marrow transplantation attempt in our patient, together with the severity of the aplastic anemia in the last 5 months prior to

Acta haemat. 62: 25–31 (1979)

Lymphocyte Subpopulations in Lymphoproliferative Diseases

Parallel Tubular Structures in Fc Receptor Bearing Lymphocytes

M. R. Halie, N. Blom, T. Kloosterman and J. W. Smit

Division of Haematology, Department of Medicine, University of Groningen, Groningen

Key Words. Fc receptors · Hodgkin lymphocytes · Parallel tubular structures

Abstract. In analogy with the data of Henkart and Henkart for normal persons, lymphocytes with parallel tubular structures (PTS or the macrotubular bundle) of patients with Hodgkin's disease were all found to bear an Fc receptor on their cell membrane (EA RFC). An explanation for this correlation of EA RFC and PTS or for the increase of EA RFC in some patients with Hodgkin's disease was not yet found. Comparison of groups of 22 normal subjects, 20 patients with Hodgkin's disease, 20 with chronic lymphatic leukemia, and 20 with non-Hodgkin lymphoma showed the EA RFC to be increased in the Hodgkin group. They were decreased in CLL patients, while mouse erythrocyte rosette-forming cells and membrane surface immunoglobulins in this group were increased. Surface Ig and EA RFC varied widely in the non-Hodgkin lymphoma group of patients.

Introduction

Cell membrane markers are now widely used to define subpopulations of lymphocytes [review *Siegal and Good* 17]. In general, T lymphocytes are characterized by their ability to form spontaneous rosettes with sheep erythrocytes (SRBC), while B lymphocytes can bind anti-immunoglobulins. A third population is found to have an Fc receptor as shown by binding of aggregated immunoglobulin or rosette-forming with (human) erythrocyte-antibody complexes. The degree of overlap of the Fc receptor-bearing lymphocytes (EA RFC) with T or B cell characteristics is not clear. In lymphocytes of mice and other animals this marker is probably present on suppressor or killer T cells [2, 3]. Human lymphocytes with an Fc receptor may in part be B lymphocytes, but the probability that they have a suppressor and/or a (non) T killer cell function is greater [14, 18, 19].

Recently *Henkart and Henkart* [7] have shown that lymphocytes of the peripheral blood from healthy persons bearing an Fc receptor contain parallel tubular structures (called the macrotubular bundle) in the cytoplasm. Earlier submicroscopic examinations have already revealed the presence of

12 Storb, R., Thomas, E. D. Weiden, P. L. Buckner C. D. Clift, R. A. Fever A. Goodell, B W., Johnson, E. L. Neimann, P. E. Sanders, J. E., and Singer J. One-hundert-ten patients with aplastic anemia (AA) treated by marrow transplantation in Seattle. Transplantn Proc. *10* 135–140 (1978)
13 Territo M. C., for The UCLA Bone Marrow Transplantation Team. Autologous bone marrow repopulation following high dose cyclophosphamide and allogeneic marrow transplantation in aplastic anemia. Br J Hemat. *36* 305–31... (1977).
14 Thomas, E. D. and Storb R., Technique for human marrow grafting. Blood *36* 507–513 (1970)
15 Thomas, E. D., Storb R. Fever A., Slichter S. J. Bryant, J. L., Buckner D. C. Neiman, P. E. Clift, R. A., Funk, D. D. and Lerner K. E. Aplastic anemia treated by marrow transplantation. Lancet *I* 284–289 (1972).
16 Thomas, E. D., Storb, R. Giblett, R. Longpre, B. Weiden, P. L. Fever A. Witherspoon, R., Clift, R. A., and Buckner C. D. Recovery from aplastic anemia following attempted marrow transplantation. Expl Hematol. *4* 97–102 (1976)
17 Tutschka, P. J., Sensenbrenner L. L., and Santos, G. W.. Marrow transplantation for aplastic anemia. Summary of Baltimore experiment. Expl Hematol. *5* suppl 2, p. 46 (1977).

Received December 9 1978
Accepted. January 8, 1979

J. Gmür Department of Internal Medicine,
University Hospital of Zürich,
CH–8091 Zürich (Switzerland)

by counting 200–300 lymphocytes under Leitz Orthoplan fluorescence microscope.

Separation of EA-RFC for Electron Microscopy
EA RFC were prepared as described above however the amount of cells was increased. 1.0 ml of the lymphocyte suspension was mixed with 1.0 ml of the EA suspension and 0.5 ml FCS.

After centrifugation for 5 min at 200 g the cells were incubated for 1 h at room temperature. Half of the supernatant was aspirated and the cell pellet was resuspended in the remaining fluid. The cell suspension was carefully layered onto ml lymphoprep and the mixture was centrifugated for 5 min at 50 g. The interphase cells and the cell pellet were collected separately and fixed for electron microscopy with 2% glutaraldehyde in 0.1 M phosphate buffer for 2 h. After fixation blocks of 1 × 1 mm were prepared from the cell pellet and rinsed in phosphate-sucrose buffer. Postfixation took place in 1% OsO for 2 h, after which the specimens were dehydrated with increasing concentrations of alcohol and embedded in Epon. Ultrathin sections were prepared on Reichert Ultramicrotome, stained with uranyl acetat and lead citrate and studied in Philips EM 201 electron microscope.

Results

In the small material of 7 patients with Hodgkin's disease so far investigated by

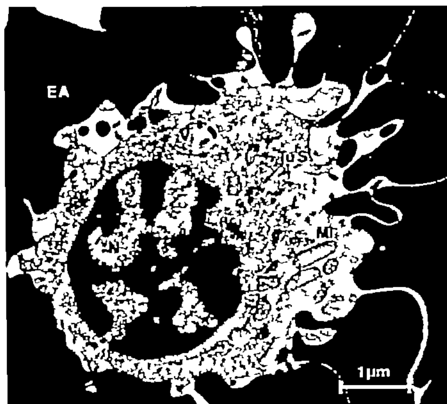


Fig. 1 Rosette-forming cell with sensitized erythrocytes (EA), showing tubular structures (TuS). N = Nucleus, MI = mitochondria, V = vacuole.

these structures in the lymphocytes of normal and diseased persons [1 4 5 9 10 13 15] In Hodgkin s disease the number of structures per cell and the total number of lymphocytes with these structures were shown to be increased [5] This increase did not correlate with the clinical or histological staging, with the impairment of cellular immunity or with raised immunoglobulin titres in the serum against cytomegalovirus or Epstein Barr virus [6]

In this study the investigation of *Henkart and Henkart* [7] on the correlation between the presence of parallel tubular structures and Fc receptors was extended to patients with Hodgkin s disease. In addition, the percentage of EA RFC of the peripheral blood was determined in patients with Hodgkin s disease and compared to the percentage in groups of normal subjects non Hodgkin lymphoma (NHL) or chronic lymphocytic leukemia (CLL) patients.

Materials and Methods

Cell membrane markers were investigated on the peripheral blood lymphocytes of 22 healthy persons (laboratory and clinical staff members) 20 patients with Hodgkin s disease, 20 with NHL and 20 patients with CLL.

Electron microscopic examination for the presence of tubular structures was performed on preparations of lymphocytes from the peripheral blood of 3 healthy persons and 7 patients with Hodgkin s disease.

Lymphocyte Isol tion

Venous blood was collected in heparin and was diluted with an equal volume of Gey s balanced salt solution in which carbonyl iron particles were suspended. The monocytes were allowed to phagocytize the carbonyl iron particles by rotating the tubes with the diluted blood at 37 °C. After this incubation the lymphocytes were isolated from the cell suspension by centrifugation on a mixture of sodium metrizoate and Ficoll (Lymphoprep Nyegaard & Co Oslo, Norway).

Demonstration of T Lymphocytes (ESRBC RFC)

SRBC were washed in TC 199 culture medium (TC 199) and a suspension of 250×10^6 cells/ml was made in this medium 0.1 ml of the lymphocyte suspension (2×10^6 cells/ml) was mixed with 0.05 ml of the SRBC suspension and with 0.05 ml fetal calf serum (FCS), which had been absorbed with SRBC. After centrifugation for 5 min at 200 *g* and incubation for at least 1 h at 4 °C, the percentage of rosette forming cells (ESRBC-RFC) was scored by counting 200 cells in a hemocytometer

Demonst ation of Lymphocytes Carrying Receptors for Mouse Erythrocytes (EMRBC RFC)

For demonstration of these receptors the same procedure was used as for demonstrating ESRBC RFC. Instead of a sheep erythrocyte suspension a mouse erythrocyte suspension of 250×10^6 cells ml was used.

Demonstration of EA RFC

EA complexes were prepared by incubating human ORh erythrocytes (R2R. erythrocytes) with human anti-D serum as described by *Zeijle m ke et l* [19]

0 1 ml of a lymphocyte suspension (4×10^6 cells/ml) were mixed with 0 1 ml of an EA-suspension (100×10^6 cells/ml) and 0 05 ml FCS. After centrifugation for 5 min at 200 *g* the mixture was incubated overnight at 4 °C. The percentage of EA RFC were scored by counting 200 cells in a hemocytometer

Demonstration of B Lymphocytes

3×10^6 lymphocytes were pelleted in a small test tube and resuspended in 3 drops of 1.20 diluted FITC-conjugated rabbit antihuman Ig antibodies (Dakopatts, Denmark). After incubation for 30 min at room temperature the cells were washed I phosphate-buffered saline (PBS). Finally the cell pellet was resuspended in a PBS-glycerol mixture (pH 8.4) and 1 drop of the suspension was placed on a microscope slide, covered and sealed. The percentage of Ig-bearing cells was determined

Table I. Percentages of lymphocytes in the peripheral blood showing different lymphocyte surface markers in groups of normal persons, patients with Hodgkin's disease, NHL or CLL

	ESRBC RFC	EMRBC-RFC	EA RFC	Ig fluor
Normal (n = 22)	75.5 ± 1.7	4.8 ± 0.8	20.0 ± 1.2	1 .5 ± 1.4
	SD 7.8	SD 2.6	SD 5._	SD 6.6
Hodgkin (n = 20)	77.2 ± 2._	2.5 ± 0.7	_5 ± 3.3	14.8 ± 1.9
	SD 9.7	SD 3.0	SD 14.9	SD 8.6
NHL (n = 20)	68 ± 5.1	3.8 ± 0.4	19.6 ± 6	29.1 ± 5.2
	SD 22.6	SD 1.8	SD 11.5	SD _3.1
CLL (n = 20)	1 .3 ± 3.1	57.2 ± 3.3	7 ± 0.6	72.9 ± 2.9
	SD 13.9	SD 14.7	SD 2.2	SD 1_.9

The values given are the mean percentages ± standard error of the mean = Number of patients SD = Standard deviation.
n = 10.

Table II. Statistical analysis (Student t test) of differences of the mean percentages of lymphocyte subpopulations between controls and patients with Hodgkin disease, NHL or CLL

		Difference with	
		normals	Hodgkin disease
Hodgkin	ESRBC-RFC	NS	
	EMRBC RFC	NS	
	EA-RFC	p = 0.05	
	Ig fluor	NS	
NHL	ESRBC-RFC	NS	NS
	EMRBC-RFC	NS	NS
	EA-RFC	NS	NS
	Ig fluor	p 0.0025	p 0.005
CLL	ESRBC-RFC	p 0.001	NC
	EMRBC RFC	p 0.01	NC
	EA-RFC	p 0.05	NC
	Ig fluor	p < 0.01	NC

NC = Not computed

tubular structures. The investigations on lymphocytes of 3 normal subjects gave the same results.

The counting of the relative percentages of lymphocytes from the peripheral blood carrying the different membrane markers in groups of healthy persons and patients with Hodgkin's disease NHL or CLL is summarized in table I.

The investigations on the healthy laboratory and clinical staff members and CLL patients are in accordance with the literature. Sheep erythrocyte, mouse erythrocyte and EA rosetting cells of NHL patients do not differ significantly ($p > 0.10$) from the normal subjects (table II), although there is a noticeable individual variation for EA RFC. The results of the immunofluorescent membrane markers of NHL patients will be published in detail elsewhere.

The lymphocytes of Hodgkin's disease patients do not differ from the normal group with respect to membrane marking by EMRBC and Ig fluorescence. The mean of EA rosetting cells is barely significantly higher ($p = 0.05$) than in the other groups. There is, however also a much larger individual variation.



Fig. 2. Detail of rosette-forming cell Many tubular structures (TuS) are present. Two centrioles (C) are localized between the tubular structures. N = Nucleus Mi = mitochondria, EA = erythrocyte sensitized with anti D antibodies.

electron microscopy the presence of the parallel tubular structures (PTS) only occurred in the EA RFC (fig 1 2) In reverse the EA rosetting cells nearly always contained parallel tubules, especially when cells were *investigated by serial sectioning*. Cells from the interphases as obtained by the separation procedure of the EA RFC did not show PTS

The concentration of rosette-forming cells in the pellet for electron microscopy showed an individual variation. Therefore, in some patients the number of lymphocytes with the tubular structures seemed to be increased as was found in our previous investigations. Also the number of structures per cell seemed to be increased but variable. The *morphology* of the structures ranged from well-defined tubules to an amorphous mass.

All observations above were irrespective of the total number of lymphocytes present in the peripheral blood of each patient or the number of lymphocytes positive for the

Immunoregulatory role of spleen thymocytes. J Immun. 112 546–554
4 Goodman, J R., Sylvester R A and Tuffanelli, D L. Virus-Like st lymphocytes of patients w th sy discoid lupus erythematosus. Ar in 79 396–402 (1973).
5 Halie, M. R., Splett Rormascano naar L, and Nieweg, H. O Parall structures in lymphocytes. I Occurr n tients with Hodgkin disease Acta hr 18–27 (1975).
6 Halie, M R., Langenhuysen, M M Gast, G. C. de, and Niew g, H O P bular structures in lymphocytes II tion with cellular immunity and cytom rus and Epstein-Barr virus antibodies F kin disease. Acta haemat. 54 82–89 197
7 Henkart, M. P and Henkart, P A Th crotubule bundle. A cytoplasmic organ characteristic f normal human blood lympho cytes bearing the F receptor Personal com mum. (Preprint, 1976).
8 Horwitz, D A. and Garrett, M A Distinc ve functional properties of human blood L lym phocytes. A comparison with T lymphocytes, B lymphocytes, and monocytes. J Immun 5 1712–1722 (1977)
9 Hovig, T Jeremic, M., and Staven, P A new type of inclusion body in lymphocytes. Scand J Haematol 6. 81–96 (1968).
10 Huhn, D Neue Organellen in peripheren Lymphozyten Dt. med. Wschr 44 2099–2100 (1969).
11 Langenhuysen, M M A. C Cazemier T Houwen, B., Brouwer Th M Halie, M. R., The, T H and Nieweg, H. O. Antibodies to Epstein-Barr virus, cytomegalovirus and Australia-antigen in Hodgkin's disease. Cancer 34 262 767 (1974).
12 Longmire, R L M Mclan, R Yelenosky R., Armstrong, S., Lang, J E., and Craddock, C. S I vitro splenic IgG synthesis in Hodgkin disease New Engl J Med 289 763–767 (1973)
1 McKenna, R. W Parkin, J Kersey J H., Ga n Peczalska, K. J Peterson, L. S., and Brunning, R D Chronic lymphoproliferative disorder with unusual clinical morphologic, ultrastructural and membrane surface marker characteristics. Am. J Med. 62. 588–596 (1977)
14 Oers, M H. J van, Zeijlemaker W P and Schellekens, P Th. A. Separation and properties f EA-rosette-forming lymphocytes in humans. Eur J Immunol. 7 143–150 (1977).
15 Payne, C. M Jones, J F Sieber O F jr and F Ignati, V A.. Parallel tubular arrays in severe combined immunodeficiency disease. An ultrastructural study of peripheral blood lymphocytes. Blood 50 55–64 (1977).
16 Rothbarth, Ph. H Hendriks-Sturkenboom, L, and Ploem, J S Identification of monocytes in suspensions of mononuclear cells. Blood 48 139–147 (1976).
17 Siegal F P and Good, R. A Human lymphocyte differentiation markers and their application to immune deficiency and lymphoproliferative diseases In Fudenberg, Disorders of lymphopoiesis and lymphoid function, pp. 355–422 (Saunders, London 1977).
18 Steinberg, A. D and Klassen, L. W Role of suppressor T cells in lymphopoietic disorders, in Fudenberg, Disorders of lymphopoiesis and lymphoid function, pp. 439–478 (Saunders, London 1977).
19 Zeijlemaker W P Oers, M. H. J van, and Eysvoogel, V P Human lymphocyte subpopulations involved in MLC and CML. Lymphocytes Isolation, fractionation and characterization. Scand J Immunol Universitetsforlaget, 5 suppl. 5, pp 143–156 (1976).

Received November 11, 1978
Accepted December 19 1978

M R. Halie, Division of Haematology
Department of Medicine, University Hospital,
Oostersingel 59 Groningen (The Netherlands)

Discussion

With regard to the increased numbers of cells with tubular structures and their morphology in patients with Hodgkin's disease, the results of the investigations were in agreement with earlier studies [5]. The nature of these structures is still not clear. The significance of their increased numbers in patients with Hodgkin's disease is also not explained.

Henkart and Henkart [7] drew our attention to their observation that the parallel tubular structures (macrotubule bundles) occur in lymphocytes with an Fc receptor. In the investigation of *McKenna et al.* [13], the lymphocytes of 3 of 4 CLL patients studied carried T lymphocyte and Fc receptor membrane markers and parallel tubular structures in the cytoplasm. Electron microscopy of cells forming EA rosettes also showed the tubular structures in lymphocytes of normal persons and of Hodgkin patients. Also in the latter group the number of structures per cell seemed to be increased. In the peripheral blood of these patients a raised number of lymphocytes with the structures was found in earlier studies [5]. It can thus be concluded that some patients with Hodgkin's disease have an increased number of cells with Fc receptors in the peripheral blood.

This was confirmed by the Fc receptor studies on the lymphocytes of healthy persons, patients with Hodgkin's disease, NHL or CLL. So far there is no explanation for this increase in EA RFC in some patients with Hodgkin's disease. First of all the question has to be answered whether these cells are indeed lymphocytes. *Rothbarth et al.* [16] have shown that the monocyte population in the peripheral blood is probably larger than assumed until now. Monocytes also carry an Fc receptor [17]. Therefore, it has to be excluded that the Fc receptor bearing cells with the parallel tubular structures are monocytes.

Secondly, when these cells are really lymphocytes, their subtype has to be established. *Brunning and Parkin* [1] suggested a T cell origin for the lymphocytes containing the parallel tubular structures. *Payne et al.* [15] found them in both T and B cells as defined respectively by sheep erythrocytes and EAC rosette forming. Analogous to the Fc receptor bearing mouse lymphocytes they could be suppressor T cells.

Speculations about the increase of suppressor T cells in the peripheral blood of a number of patients with Hodgkin's disease include that they can be involved trying to shut off the immunoglobulin production against CMV or EBV. Since serum levels of antibodies against those viruses are raised in a similar patient group [11], countermanding of this production could lead to a raised number of EA RFCs with suppressor T cell function. The same connection could exist with the antibody production against the patients own lymphocytes [12]. Finally, with the function of (non)T killer cells [19] in mind, they could be reacting against the tumor itself.

References

1 Brunning, R. D. and Parkin, J.: Ultrastructural studies of parallel tubular arrays in human lymphocytes. Am. J. Path. 78: 59-70 (1975).
2 Cantor, H. and Weissman, I.: Development and function of subpopulations of thymocytes and T lymphocytes. Progr. Allergy, vol. 20, p. 1 (Karger, Basel 1975).
3 Gershon, R. K., Lance, E. M. and Kondo, K.

Immunoregulatory role of spleen localizing thymocytes. J Immun. *112*. 546–554 (197

4 Goodman, J R., Sylvester R A., Tal N and Taffanelli, D L. Virus-like structures in lymphocytes f patients with system and discoid lupus erythematosus. Ann Intern Med 79 396–402 (1973).

5 Halie, M. R., Splett Romascano, M Molenaar L, and Nieweg, H. O.. Parallel tubular structures in lymphocytes. I. Occurrence in patients with Hodgkin disease. Acta haemat 54 18–27 (1975).

6 Halie, M R., Langenhuysen, M. M. A C Gast, G. C. de, and Nieweg, H. O Parallel tubular structures in lymphocytes. II Correlation with cellular immunity and cytomegalovirus and Epstein-Barr virus antibodies in Hodgkin disease. Acta haemat 54 82–89 (1975)

7 Henkart, M P and Henkart, P A The macrotubule bundle. A cytoplasmic organell characteristic of normal human blood lymphocytes bearing the F receptor Personal communi. (Preprint, 1976).

8 Horwitz, D A. and Garrett, M. A.. Destinctive functional properties of human blood L lymphocytes. A comparison with T lymphocytes, B lymphocytes, and monocytes J Immun 5 1712 1722 (1977).

9 Hovig, T Jeremic, M and Staven, P A new type of inclusion body in lymphocytes. Scand J Haematol 6 81–96 (1968).

10 Huhn, D.. Neue Organellen in peripheren Lymphocyten? Dt med. Wschr 44 2099–2100 (1968).

11 Langenhuysen, M. M A C Carremer T Hourwen, B., Brouwer Th M Halie, M R The, T H., and Nieweg, H O.. Antibodies to Epstein-Barr virus, cytomegalovirus and Australia-antigen in Hodgkin disease Cancer 34 262 267 (1974).

12 Longmire, R L McMillan, R Yelenosky R Armstrong, S., Lang, J E., and Craddock, C. S *I vitro* splenic IgG ynthesis in Hodgkin's disease New Engl. J Med. 289 763–767 (1973).

13 McKenna, R. W Parkin, J Kersey J H, Gajl-Peczalska, K. J Peterson, L. S and Brunning, R. D Chronic lymphoproliferative disorder with unusual clinical morphologic, ultrastructural and membrane surface marker characteristics. Am. J Med. 62. 588–596 (1977).

14 Oers, M. H J van Zeijlemaker W P and Schellekens, P Th. A.. Separation and properties of EA-rosette-forming lymphocytes in humans. Eur J Immunol. 7 143–150 (1977).

15 Payne, C. M., Jones, J F Sieber O. F jr and Fulginiti, V A.. Parallel tubular arrays in severe combined immunodeficiency disease An ultrastructural study of peripheral blood lymphocytes. Blood 50 55–64 (1977).

16 Rothbarth, Ph. H Hendriks-Sturkenboom, L, and Ploem, J S.. Identification of monocytes in suspensions of mononuclear cells. Blood 48 139–147 (1976).

17 Siegal, F P and Good, R. A Human lymphocyte differentiation markers and their application to immune deficiency and lymphoproliferative diseases, in Fudenberg, Disorders of lymphopoiesis and lymphoid function, pp 355–422 (Saunders, London 1977).

18 Steinberg, A. D. and Klassen, L. W Role of suppressor T cells in lymphopoietic disorders, in Fudenberg, Disorders of lymphopoiesis and lymphoid function, pp. 459–478 (Saunders, London 1977).

19 Zeijlemaker W P Oers, M. H. J van, and Eysvoogel, V P Human lymphocyte subpopulations involved in MLC and CML. Lymphocytes. Isolation, fractionation and characterization. Scand. J Immunol Universitetsforlaget, 5 suppl 5 pp. 143–156 (1976).

Received November 11, 1978
Accepted December 19 1978

M R Halie, Division of Haematology
Department of Medicine, University Hospital,
Oostersingel 59 Groningen (The Netherlands)

Acta haemat. 62 32–40 (1979)

Acid-Base Balance Changes and Erythropoietin Production in the Early Stages of Hypoxia or after $CoCl_2$ Treatment in the Rabbit

M. Vittoria Ambrosini, Giovanni B. Principato, Elvio Giovannini, Gigliola Grassi and Piero Toraldo

Institute of General Biology, Faculty of Medicine and Surgery; Chair of General Physiology, Faculty of Sciences, University of Perugia, and Centre of Nuclear Medicine, Regional General Hospital, Perugia

Key Words. Acid-base balance · Anemic hypoxia · Hypobaric hypoxia · $CoCl_2$ treatment · Blood lactate level · Erythrocyte 2,3-DPG · Erythropoietin

Abstract. Acid base balance changes as well as rises in erythropoietin (Ep) levels were studied in rabbits during the first 12 h of either anemic or hypobaric hypoxia and following $CoCl_2$ treatment. Bleeding and $CoCl_2$ administration lead to an early and transient decrease in blood pH associated with a rise in blood lactate. Hypobaric exposure causes alkalosis and lactate increase, both persisting at 12 h. Both hypobarism and $CoCl_2$ treatment lead to decrease in pCO_2 which is far greater than after bleeding. Only hypobaric exposure produces a rise in erythrocyte 2,3-DPG; in addition, it leads to far higher plasma Ep levels than bleeding or $CoCl_2$ treatment. It is unlikely that pCO_2 lowering plays a determinant role in Ep production, while a possible enhancing effect of blood pH other than Bohr effect remains quite hypothetic. Ep production seems to be actually correlated with blood lactate increases, in agreement with a regulatory role of this metabolite on Ep production suggested by other authors.

It is well known that bleeding, exposure to a hypoxic environment and $CoCl_2$ treatment lead to an increased production of erythropoietin (Ep), the humoral factor which acts upon the erythroblastic tissue [7]. The response to anemic or hypoxic hypoxia depends on a reduced delivery of oxygen to the kidney, the organ which plays a fundamental role in the Ep production; instead, cobalt stimulates it by causing a renal histotoxic hypoxia [5]. The degree of tissue hypoxia may also be affected by other mechanisms. Studies concerning hypoxia in man [12] and $CoCl_2$ treatment in rats [11] have shown that a rise in Ep production follows changes in acid-base balance. In particular it occurs in respiratory alkalosis with increase in blood pH and decrease in pCO_2; this leads to an increased affinity of hemoglobin (Hb) for oxygen (Bohr effect) with decrease in p50 and hence to a reduced oxygen supply to the tissues. Similar changes

in Hb affinity for oxygen, associated with polycythemia, have also been observed in patients with hemoglobinopathies [2]. Therefore, it was thought that the increases in Hb affinity for oxygen could significantly affect the Ep production following hypoxia [17]. On the contrary *Miller* [10] and *Miller et al.* [13] have observed that the rise in Ep level seems more directly correlated to an increase in pH and/or to a decrease in pCO_2, hence, they suggested that such variations played a greater role in Ep production than was previously supposed.

On this basis, it seemed of interest to study Ep production and acid-base balance changes in rabbits subjected to various treatments enhancing Ep production (bleeding, hypobaric hypoxia, $CoCl_2$ treatment) in order to obtain further data suitable for detecting the parameter(s) whose variations led to increased Ep levels.

It also seemed useful to measure the blood lactate titers, because lactic acid production, as well as that of Ep, depends on the degree of hypoxia. The former may affect the acid-base balance; moreover blood lactate increases in rats following hypobaric exposure or $CoCl_2$ treatment and it is thought to play a regulatory role in Ep production [15, 16].

Finally the erythrocyte 2,3-DPG level was measured, because it is affected by the changes in acid-base status that occur during hypoxia [1, 2, 9]; moreover it early increases in rats following hypoxic exposure [18] or bleeding [13] as plasma Ep levels are rising.

Materials and Methods

Rabbits of both sexes were used, approximately 6 months old, weighing about 3 kg and fed with commercial rabbit pellets and tap water *ad libitum*. The various experiments were performed with groups of animals which were subjected respectively to bleeding (20 ml of blood/kg of body weight by intracardiac puncture), hypobaric exposure (0.42 atm for 12 h in a decompression chamber) or $CoCl_2$ treatment (250 μmol/kg of body weight, given as a single intramuscular injection of 0.25 *M* $CoCl_2 \cdot 6H_2O$ in bidistilled water).

For each of the experimental conditions the blood pH, pCO_2 and lactate level, erythrocyte 2,3-DPG and blood Hb concentration were measured using a total of 3 groups of 6 rabbits. These parameters were more closely evaluated during the first hours of experiment, when metabolic changes would have appeared and the reaction mechanisms to the applied stress would have started. The various determinations were performed on small arterial blood samples (about 1.5–2 ml) taken anaerobically at successive times (0, 0.75, 1.5, 3, 4.5, 6, 9, 12 h) by intracardiac puncture and using heparinized plastic syringes (needle No. 12); the overall handling time required for a single puncture was usually limited to 10 sec. It was thus possible to follow in each group of animals the evolution of the phenomena studied with time. In order to evaluate the possible influence of stress factors (lung hyperventilation, muscle tension) on the acid-base status due to repeated cardiac punctures in unanesthetized animals, an untreated group of 6 rabbits was subjected, as a control, to the same samplings and measurements.

In addition, plasma Ep levels were evaluated in the above-stated experimental conditions using 3 further groups of 6 rabbits each. Blood was collected (4–5 ml/animal) at 0, 6, 9 and 12 h by intracardiac puncture; we have stated such time intervals since previous studies carried out in one of our Institutes ha[ve] shown in the rabbit a rise in plasma erythrostimulant activity [wi]th [a] peak at 9 h following both bleeding [8] and $CoCl_2$ treatment [9]. Likewise, increased Ep titers were noted in rabbits after 8 h of hypobaric hypoxia [14]. Blood samples were centrifuged at 3,000 rpm for 10 min, plasma samples thus obtained from each experimental group prior to and at each time after the treatment were pooled and stored at −20 °C until assayed for erythrostimulant activity.

Analytical Methods

The pH and pCO_2 [v]alues of whole arterial blood were measured at 37 °C using a Gas Analy-

zer Radiometer BMS3 Blood lactate and erythrocyte 2,3-DPG levels were determined by enzymatic assay initiating from extracts obtained from blood samples (0.5 ml) immediately deproteinized by 0.6 *N* $HClO_4$. The technique described by *Drewes* [4] and the Boehringer Biochemica test were respectively followed in both cases the measures were based on the NADH spectrophotometric determination at 340 nm (Beckman DK 2A apparatus). The Hb was measured as cyanmethemoglobin by evaluating spectrophotometrically the absorbance at 540 nm.

Erythrostimulant activity was evaluated using the polycythemic mouse bioassay according to a modification of the method initially described by *Cotes and Bangham* [3] Female Swiss mice (20–25 g) were made polycythemic by exposure to 0.42 atm for 16 days in a hypobaric chamber the mice were removed for 5 h for each day at which time they were given water and food. On the 7th and 8th posthypoxic days, the mice were injected subcutaneously with divided doses of the total sample (1 ml) to be tested (isotonic saline, standard Ep in saline or rabbit plasma). Each preparation was assayed in a group of 10–15 mice. On the 9th posthypoxic day each animal was given intraperitoneally 0.5 μCi of ^{59}Fe-citrate in saline (0.5 ml) 72 h later the mice were killed by decapitation under light ether anesthesia and blood (0.8–1 ml) collected animals showing a hematocrit of less than 56% were discarded. Blood was then counted on a Packard Tri-Carb scintillation spectrometer the percent ^{59}Fe uptake into newly formed red cells was determined (blood volume was assumed to be 7% of the body weight) and converted to the equivalent units of the International Reference Preparation (IRP) of Ep. For this purpose a log dose-response regression line was obtained using Ep standard B (Connaught Medical Research Lab Toronto).

Results

The results obtained in the course of the various experiments were statistically assessed by variance analysis. For each of the parameters studied, the significance of the variations in time was evaluated in addition, comparisons among effects of different treatments were sometimes performed. We considered variations caused by both the treatment and the individual variability whenever measurements on the same animals at successive times were carried out;

Table I. Blood pH levels[1] in rabbits prior to and following bleeding (A), exposure to hypobaric hypoxia (B) or $CoCl_2$ treatment (C) and in an untreated control group

Time, h	A	B	C	Control
0	7.47 ± 0.012	7.44 ± 0.018	7.45 ± 0.022	7.43 ± 0.018
0.75	7.39 ± 0.024	7.46 ± 0.018	7.39 ± 0.017[2]	7.42 ± 0.021
1.5	7.41 ± 0.022[3]	7.50 ± 0.029[3]	7.35 ± 0.017[2]	7.44 ± 0.023
3	7.43 ± 0.016[2]	7.52 ± 0.022[2]	7.43 ± 0.025	7.45 ± 0.030
4.5	7.44 ± 0.016	7.49 ± 0.019	7.46 ± 0.027	7.44 ± 0.023
6	7.44 ± 0.014	7.49 ± 0.017	7.48 ± 0.023	7.43 ± 0.030
9	7.47 ± 0.018	7.50 ± 0.011[2]	7.47 ± 0.028	7.45 ± 0.026
12	7.46 ± 0.018	7.49 ± 0.015[2]	7.48 ± 0.027	7.44 ± 0.023

1 Mean values ± standard error of 6 measurements.
Significantly different ($p < 0.05$) from initial.
Significantly different ($p < 0.01$) from initial.
Comparisons with initial values were carried out by Duncan's test and evaluating the individual variability

this method allowed a reduction of the experimental error. Duncan's test was used to compare initial and successive groups of data including the same number of measurements. Results and statistical evaluations are reported in the tables.

Regarding the acid-base balance, both bled and $CoCl_2$ treated rabbits show a significant decrease in the blood pH within 0.75 h; such a parameter returns to normal levels within 4.5 and 3 h, respectively. On the contrary, exposure to hypoxia leads to an alkalotic state with significant increases in the pH within 0.75–1.5 h, that persist at 12 h (table I). pCO_2 significantly decreases within 0.75 h following a bleeding or hypobaric exposure and within *0.75–1.5 h following* $CoCl_2$ treatment; such lower-than-normal values persist at 12 h. As to this parameter, we thought it useful to carry out a statistical comparison among treatments; *this shows that, at least from 4.5 h*, pCO_2 levels following hypobaric hypoxia or $CoCl_2$ treatment are significantly lower ($p < 0.01$) than after bleeding. Moreover, no significant difference in pCO_2 levels is detectable between hypobarism-exposed and $CoCl_2$ treated rabbits, at *least* from 4.5 to 9 h (table II).

Blood lactate values rapidly increase in the various groups of animals; they are significantly higher than initial levels within 0.75 h following bleeding or hypobaric hypoxia and within 0.75–1.5 h after $CoCl_2$ administration. The examined parameter returns to zero-time values within 3 h in bled rabbits and within 4.5 h in those $CoCl_2$ treated, while it still shows significantly higher than initial titers at 12 h after hypobaric exposure (table III).

The erythrocyte 2,3-DPG level does not show within the considered time limits, significant variations in bled or $CoCl_2$ treated rabbits. In animals subjected to hypobaric hypoxia this parameter instead shows increases which become *significant from 4.5* to 6 h after the beginning of the treatment (table IV).

Table II. Blood pCO_2 (mm Hg) levels in rabbits prior to and following bleeding (A), exposure to hypobaric hypoxia (B) or $CoCl_2$ treatment (C) and in an untreated control group

Time, h	A	B	C	Control	Comparisons[1] among A, B, C
0	32.3 ± 0.386	32.2 ± 0.660	33.5 ± 1.525	31.8 ± 1.061	NS
0.75	25.9 ± 0.327[2]	25.1 ± 1.000[2]	31.5 ± 1.299	32.0 ± 1.232	C vs. A, B $p<0.01$ A vs. B NS
1.5	26.0 ± 1.207[2]	22.1 ± 0.957[2]	28.0 ± 0.951	29.3 ± 1.818	B vs. A, C $p<0.01$ A vs. C NS
3	28.1 ± 0.963	19.5 ± 0.837[2]	4.4 ± 1.969[2]	29.5 ± 1.982	B vs. A, C $p<0.01$ A vs. C NS
4.5	27.5 ± 0.870[2]	17.7 ± 0.620[2]	18.1 ± 1.726	29.7 ± 1.778	A vs. B, C $p<0.01$ B vs. C NS
6	27.6 ± 0.791	18.4 ± 0.407[2]	17.7 ± 1.544	29.9 ± 1.727	A vs. B, C $p<0.01$ B vs. C NS
9	77.2 ± 1.864	16.8 ± 1.392[2]	18.3 ± 1.453	30.7 ± 2.245	A vs. B, C $p<0.01$ B vs. C NS
12	28.3 ± 0.874	15.5 ± 0.865	20.3 ± 1.938	30.3 ± 1.978	A vs. B, C $p<0.01$ B vs. C $p<0.05$

Mean values ± standard error of 6 measurements.

[1] Carried out by variance analysis.

[2] Significantly different ($p < 0.01$) from initial.

Comparisons carried out by Duncan's test and evaluating the individual variability.

NS = Not significant.

Table III. Blood lactate (mmol/100 ml) levels[1] in rabbits prior to and following bleeding (A), exposure to hypobaric hypoxia (B) or $CoCl_2$ treatment (C) and in an untreated control group

Time, h	A	B	C	Control
0	0.45 ± 0.037	0.56 ± 0.069	0.49 ± 0.029	0.46 ± 0.031
0 75	0.90 ± 0.082[3]	1.02 ± 0.157[3]	0.54 ± 0.029	0.52 ± 0.053
1.5	0 71 ± 0.030[3]	0.97 ± 0.110[3]	0 61 ± 0.052[3]	0.48 ± 0.045
3	0.60 ± 0 086	0 82 ± 0.086[3]	0.62 ± 0.051[3]	0.53 ± 0.061
4.5	0.46 ± 0 041	0.96 ± 0.100[3]	0.56 ± 0.024	0.52 ± 0.060
6	0.50 ± 0.049	0 85 ± 0.059[3]	0.54 ± 0 040	0.51 ± 0.058
9	0.46 ± 0.064	0.93 ± 0.071[3]	0.52 ± 0.054	0.54 ± 0.056
12	0 42 ± 0 030	1.00 ± 0.117[3]	0.55 ± 0.042	0.50 ± 0.055

[1] Mean values ± standard error of 6 measurements.
[2] Significantly different ($p < 0.05$) from initial.
[3] Significantly different ($p < 0.01$) from initial.
Comparisons with initial values were carried out by Duncan's test and evaluating the individual variability

Table IV Erythrocyte 2,3-DPG (μmol/g Hb) levels[1] in rabbits prior to and following bleeding (A), exposure to hypobaric hypoxia (B) or $CoCl_2$ treatment (C) and in an untreated control group

Time, h	A	B	C	Control
0	32.8 ± 1.339	31.5 ± 1 068	33 6 ± 2.258	30.0 ± 1.577
0 75	31.6 ± 1 152	32.0 ± 1 132	33.5 ± 1.984	30.3 ± 1.531
1.5	30.7 ± 1 005	32.5 ± 1 413	33 1 ± 1 472	29 1 ± 1.613
3	32.0 ± 1 086	30.9 ± 0.91	33 7 ± 1.523	31.0 ± 1 466
4.5	31.2 ± 0.858	33 4 ± 1 479	33 4 ± 1.665	30.8 ± 1.641
6	30.4 ± 1.305	34.5 ± 1.230[2]	32.0 ± 1.919	32.2 ± 2.396
9	32.5 ± 0.557	35.2 ± 1 167[2]	32.2 ± 1 796	30 7 ± 2.499
12	32.0 ± 1.255	35.3 ± 1 133[3]	31 6 ± 2.123	31 7 ± 2.108

[1] Mean values ± standard error of 6 measurements
[2] Significantly different ($p < 0.05$) from initial.
Significantly different ($p < 0.01$) from initial
Comparisons with initial values were carried out by Duncan's test and evaluating the individual variability

Blood Hb concentration rapidly decreases following a bleeding, reaching within 1.5 h values which no longer show significant differences among them. Cobalt treatment leads instead to a significant blood Hb increase within 0 75 h Hb values return to zero-time levels within 9 h. Hypoxia-exposed rabbits show no significant variation in times as regards the examined parameter (table V)

The animals of the control group merely subjected to repeated blood samplings, do not show significant changes in times regarding the various parameters studied by us (table I–V)

Plasma Ep levels significantly increase

Table V. Blood hemoglobin concentration (g/100 ml) values in rabbits prior to and following bleeding (A), exposure to hypobaric hypoxia (B) or $CoCl_2$ treatment (C) and in an untreated control group

Time, h	A	B	C	Control
0	12.38 ± 0.389	11.71 ± 0.523	12.20 ± 0.501	11.92 ± 0.633
0.75	9.78 ± 0.577[1,2]	11.93 ± 0.579	13.74 ± 0.310[2]	11.98 ± 0.704
1.5	9.10 ± 0.461	11.41 ± 0.561	13.75 ± 0.296[2]	12.00 ± 0.415
3	8.87 ± 0.339[2]	11.37 ± 0.603	13.63 ± 0.460[2]	11.66 ± 0.617
4.5	8.86 ± 0.347[2]	11.35 ± 0.673	13.03 ± 0.552[2]	11.58 ± 0.512
6	8.44 ± 0.466[2]	11.12 ± 0.756	12.95 ± 0.506[2]	11.37 ± 0.605
9	8.31 ± 0.385	11.23 ± 0.581	12.23 ± 0.387	11.43 ± 0.458
12	7.92 ± 0.404	10.88 ± 0.789	12.18 ± 0.287	11.11 ± 0.718

Mean values ± standard error of 6 measurements
[1] Significantly different ($p < 0.05$) from 12 h value
[2] Significantly different ($p < 0.01$) from initial.
Comparisons with initial or 12 h values were carried out by Duncan's test and evaluating the individual variability

Table VI. Plasma erythropoietin (Ep) levels in rabbits prior to and following bleeding (A), exposure to hypobaric hypoxia (B) or $CoCl_2$ treatment (C) bioassay in exhypoxic polycythemic mice

Time h	A		B		C		Comparisons among A, B, C
	% 59Fe uptake (72 h) in RBC[1]	IRP units of Ep/ml	% 59Fe uptake (72 h) in RBC[1]	IRP units of Ep/ml	% 59Fe uptake (72 h) in RBC[1]	IRP units of Ep/ml	
0	1.35 ± 0.104 (10)	ND	1.20 ± 0.133 (10)	ND	1.09 ± 0.090 (12)	ND	NS
6	7.61 ± 1.723 (12)	0.26	23.31 ± 3.146[2] (12)	1.19	8.90 ± 1.408 (15)	0.28	B vs. A, C. $p < 0.01$ A vs. C NS
9	8.65 ± 1.737[2] (12)	0.29	29.85 ± 619 (15)	2.23	9.40 ± 1.590[2] (14)	0.31	B vs. A, C $p < 0.01$ A vs. C NS
12	7.94 ± 1.690[2] (9)	0.27	30.89 ± 2.278 (12)	2.47	10.40 ± 1.522 (10)	0.34	B vs. A, C $p < 0.01$ A vs. C NS

Isotonic saline, % 59Fe uptake (72 h) in RBC 0.69 ± 0 075 (8).
Mean values ± standard error the number of assays is indicated in parentheses.
Carried out by variance analysis.
Significantly different ($p < 0.01$) from initial (variance analysis).
ND = Not detectable NS = not significant.

following the various treatments however Ep titers are far higher in the rabbits subjected to hypobaric hypoxia than in the animals of the other groups (7/9-fold at 12 h, considering the Ep units). These show no significant difference in plasma Ep levels when compared to each other at the various time intervals (table VI).

Discussion

Our present findings concerning acid-base balance show that the changes in blood pH following the various treatments occur in opposite directions and are *different in* duration. The transient pH decreases after bleeding or $CoCl_2$ administration are likely to be the result of increased lactic acid production. In fact, a *rise in blood lactate titers* occurs almost at the same time as pH changes. The bled and, more markedly the $CoCl_2$ treated rabbits, show lower than normal pCO_2 *values persisting after the blood* pH has returned to initial levels. This fact may be due, at least in part, to increased respiratory frequency observed in these animals; *however* pCO_2 *is probably also af*fected by metabolic changes associated with the experimental conditions, mainly following $CoCl_2$ administration (histotoxic hypoxia). *The animals subjected to hypobaric* exposure display an intense and evident lung hyperventilation and, in spite of a persistent increase in blood lactate level, develop a respiratory alkalosis with a profound decrease in pCO_2.

As mentioned above, *Miller* [10] suggested a possible involvement of blood pH and/or pCO_2 as regulators of Ep production through mechanisms other than changes in the Hb affinity for oxygen. Our present findings lead us to believe it unlikely that pCO_2 lowering plays a determinant role in Ep production, since the different decreases in pCO_2 and increases in Ep occurring after the various treatments display no reasonable correlation. In fact, rabbits subjected to hypobaric hypoxia or $CoCl_2$ administration show for several hours pCO_2 decreases of the same magnitude, while the former show plasma Ep levels even 7 fold greater than the latter (12 h). In addition, beginning from a few hours after the treatment, bled and $CoCl_2$ treated rabbits show widely different pCO_2 levels but, on the contrary, reach plasma Ep titers without significant differences. *The changes in blood* pH observed by us occur, as stated above, in opposite directions and with different durations following the various treatments; *moreover, high and increasing plasma* Ep levels are associated in time with alkalosis (hypobarism), while far lower Ep titers are observed after temporary decreases in blood *pH (bleeding and* $CoCl_2$ *treatment).* Such a correlation could lead one to suppose, bearing again in mind the suggestion of *Miller* [10], the existence of a mechanism through *which blood pH increase, rather than* pCO_2 lowering, may potently enhance Ep production; however, the existence of such a mechanism still remains quite hypothetic.

Lactic acid production depends, as it is well known, on the degree of hypoxia. In our groups of experimental animals blood lactate levels show increases differing from one another in magnitude as well as in duration. After a bleeding such an increase seems to be linked with the hypovolemic shock following the treatment. In fact, lactate values return to control levels when blood Hb concentration tends toward steady values after the rapid initial decrease. In addition, lactate increase following $CoCl_2$ administration seems to be associated with the hemoconcentration which occurs early in this condition. Such a phenomenon has been studied in a recent report of our [6]; as regards the present findings, it is evidenced by the increase in blood Hb values occurring within the first 9 h. Therefore, it is likely that in both bled and $CoCl_2$ treated rabbits hypovolemia leads to a lowering in

the cardiac output with a reduction of the oxygen delivery to the tissues. Such an additional hypoxia may enhance lactic acid production. The prolonged lactate elevation following hypobaric exposure suggests that in the rabbit this treatment produces, within the considered time limits, a greater and more persistent hypoxia than bleeding or cobalt administration such an effect may be additionally strengthened by increased Hb affinity for oxygen due to the alkalosis (Bohr effect).

Since both lactic acid and Ep production depend on the degree of tissue hypoxia, it is reasonable to search for a correlation between blood lactate changes and Ep production. Our findings, in fact, show high and increasing Ep titers following hypobaric exposure associated with persistently high levels of blood lactate this substance shows, on the contrary an early and transient increase following a bleeding or CoCl treatment, both leading to far smaller increases in Ep than hypobaric hypoxia. *Rodgers et al.* [15 16] found that cobalt as well as hypobarism produces an increase in blood lactate levels of rats they suggested that the effects of these treatments on kidney Ep production may be modulated by a lactate-induced modification in renal cortical adenylate cyclase activity since lactate stimulates *in vitro* this enzyme and, in addition, lactate administration causes a profound erythropoietic effect. Therefore, our findings seem to substantiate the existence of an important regulatory role of lactate in Ep production following the various hypoxic stimuli, explainable through the mechanism described by the above-mentioned authors.

It is well known that an increase in erythrocyte 2,3-DPG during hypoxia leads to a reduction in Hb affinity for oxygen, hence to a better delivery of oxygen to the tissues such a mechanism is thought to play an important role in reducing Ep response [17] Our findings agree with this model In fact, increased erythrocyte 2,3-DPG levels appear within 12 h, but only following hypobaric hypoxia, leading to far higher Ep levels than bleeding or $CoCl_2$ administration. Moreover unlike other treatments, hypobarism causes a shift in blood pH towards alkaline values therefore, such a condition appears once again to be a determinant factor for an increase in erythrocyte 2,3-DPG [1 2, 19]

Our further studies concerning factors whose changes may affect Ep production in response to erythropoietic stimuli are in progress.

Acknowledgements

The authors thank Mr *Antonio Buracci* (Institute of Labour Medicine, Faculty of Medicine and Surgery Perugia University) for his excellent technical assistance.

References

1 Bellingham, A. J Detter J C and Lenfant, C Regulatory mechanisms of hemoglobin oxygen affinity in acidosis and alkalosis. J clin. Invest. *50* 700–704 (1971).

2 Bellingham, A J Eritrocita ed adattamento all'ipossia anemica, in Garby Anemia ipossia, pp. 103–123 (Pensiero Scientifico, Roma 1975).

3 Cotes, P M. and Bangham, D R. Bioassay of erythropoietin in mice made polycythaemic by exposure t air at reduced pressure. Nature, Lond *191* 1065–1067 (1961)

4 Drewes, P A. Carbohydrate derivatives and metabolites; in Henry Cannon and Winkelman, Clinical chemistry principles and tech-

nics, pp 1327–1334 (Harper & Row Hagerstown 1974).
5 Fisher J W and Langston, J W The influence of hypoxemia and cobalt on erythropoietin production in the isolated perfused dog kidney Blood *29* 114–125 (1967)
6 *Giovannini, E., Principato, G B., Ambrosini, M V.*, Grassi, G and dell Agata, M Early effects of cobalt chloride treatment on certain blood parameters and on urine composition. J Pharmac. exp Ther (accepted for publication, 1978).
7 Krantz, S B and Jacobson, L. O. Erythropoietin *and the regulation of erythropoiesis* (University of Chicago Press, Chicago 1970).
8 Liotti, F S e Giovannini, E. Studi e ricerche in tema di rapporti tra rene e milza nel controllo dell eritropoiesi Haematologica *55* 681–697 (1970)
9 Liotti, F S., Biagioni, M e Migliorini Bruschelli, G. *Il ruolo della milza nella reticolocitosi* da cobalto. Riv Biol. *64* 321–354 (1971)
10 Miller M E The interaction between the regulation of acid-base and erythropoietin production. Blood Cells *1* 449–465 (1975)
11 Miller M. E. Howard, D., Stohlman, F., jr and Flanagan, P. Mechanism of erythropoietin production by cobaltous chloride. *Blood 44* 339–346 (1974).
12 Miller M E., Rörth, M. Parving, H H. Howard, D Reddington, I., Valeri, C. R., and Stohlman, F., jr pH effect on erythropoietin response to hypoxia. New Engl. J Med. *288* 706–710 (1973)
13 *Miller M. E. Rörth, M. Stohlman, F jr* Valeri, C. R., Lowrie, G Howard, D and McGilvray N The effects of acute bleeding on acid-base balance, erythropoietin (Ep) production and *in vivo* p 50 Br J Haemat. *33* 379–385 (1976).
14 Paulo, L. G., Roh, B. L., and Fisher J W Antagonism of erythropoietin production in rabbits by atropine. Proc. Soc. exp. Biol. Med. *139 207–210 (1972).*
15 Rodgers, G M., Fisher G W and George, W J. Lactate stimulation of renal cortical adenylate cyclase: a mechanism for erythropoietin production following cobalt treatment of hypoxia. J Pharmac. exp. Ther *190:* 542–550 (1974).
16 *Rodgers, G M Fisher J W and George, W* J Renal cyclic AMP accumulation and adenylate cyclase stimulation by erythropoietic agents. Am. J Physiol. *229* 1387–1392 (1975).
17 Rörth, M Ipossia, affinità dell'eritrocita per l'ossigeno e produzione di eritropoietina, in Garby Anemia e Ipossia, pp. 145–160 (Pensiero Scientifico, Roma 1975).
18 Schooley J C. and Mahlmann, L. J. Hypoxia and the initiation of erythropoietin production. Blood Cells *1* 429–448 (1975)
19 Torrance, J Jacobs, P., Restrepo, A., Esbach, J., Lenfant, C., and Finch, C. A. Intraerythrocytic adaptation to anemia, New Engl. J Med. *283 165–169 (1970).*

Received. September 1 1978
Accepted November 15, 1978

Dr Maria Vittoria Ambrosini,
Istituto di Biologia generale dell'Università,
Via del Giochetto I-06100 Perugia (Italia)

Acta haemat. 62: 41–44 (1979)

Zinc Levels of Plasma, Erythrocyte, Hair and Urine in Homozygote Beta Thalassemia

Ülker Doğru, Ayten Arcasoy and Ayhan O. Çavdar

Hematology Division of the Department of Pediatrics, Faculty of Medicine, University of Ankara, Ankara

Key Words. Thalassemia · Zinc

Abstract. The zinc levels of plasma, erythrocytes, hair and urine were determined by atomic absorption spectrophotometer technique in 20 homozygous β-thalassemic and 20 control children. Plasma, erythrocyte and hair zinc levels in the diseased children were found to be much lower than those of controls. The difference was statistically highly significant. Along with other factors, zinc deficiency was throught to have an important role in the retardation of growth and sexual development seen in the patients with homozygous β-thalassemia. The increased urinary zinc excretion suggests that the factor responsible for zinc deficiency might be the loss of the element through the kidneys.

One of the most outstanding clinical features of homozygous β-thalassemia is growth retardation [1–3]. The syndrome described by Prasad, which is characterized by geophagia, iron deficiency anemia, hepatosplenomegaly, hypogonadism and decreased serum level of zinc, is clinically identical with the homozygous β-thalassemia associated with sexual detention and growth retardation. Because of this clinical similarity it has been implicated that the patient with thalassemia major could have secondary zinc deficiency [4, 5].

Although the serum zinc level is known to be low in patients with homozygous β-thalassemia [4, 5], the quantity of this trace element in the urine, erythrocytes and hair has not been sufficiently investigated in this disease.

Material and Method

The study has been carried out on 20 homozygous β-thalassemic patients, 10 male and 10 female. Their ages varied between 3 and 20 years. In all cases diagnosis of thalassemia was established by clinical, hematologic, electrophoretic and genetic workup. Standard methods were applied for routine hematological tests. Hemoglobin A_2 was measured by cellulose acetate electrophoresis and fetal hemoglobin by alkali denaturation tests [6].

The control group of 20 cases comprised 10 male and 10 female nonthalassemic children selected from similar age range and socioeconomical class as the patients.

nics, pp 1327–1334 (Harper & Row Hagerstown 1974).
5 Fisher J W and Langston, J W. The influence of hypoxemia and cobalt on erythropoietin production in the isolated perfused dog kidney Blood *29* 114–125 (1967)
6 Giovannini, E., Principato, G B., Ambrosini, M. V Grassi, G., and dell Agata, M. Early effects of cobalt chloride treatment on certain blood parameters and on urine composition. J Pharmac. exp. Ther (accepted for publication, 1978).
7 Krantz, S. B and Jacobson, L. O Erythropoietin and the regulation of erythropoiesis (University of Chicago Press, Chicago 1970)
8 Liotti, F S. e Giovannini, E. Studi e ricerche in tema di rapporti tra rene e milza nel controllo dell eritropoiesi. Haematologica *55* 681–697 (1970)
9 Liotti F S., Biagioni, M e Migliorini Bruschelli, G Il ruolo della milza nella reticolocitosi da cobalto Riv Biol. *64* 321–354 (1971)
10 Miller M. E. The interaction between the regulation of acid-base and erythropoietin production. Blood Cells *1* 449–465 (1975).
11 Miller M. E., Howard, D., Stohlman, F., jr., and Flanagan, P Mechanism of erythropoietin production by cobaltous chloride. Blood *44* 339–346 (1974).
12 Miller M. E. Rörth, M. Parving, H H. Howard, D., Reddington, I Valeri, C. R., and Stohlman, F jr pH effect on erythropoietin response to hypoxia. New Engl. J Med. *288* 706–710 (1973)
13 Miller M E., Rörth, M. Stohlman, F jr Valeri, C. R. Lowrie, G Howard, D and McGilvray N. The effects of acute bleeding on acid-base balance, erythropoietin (Ep) production and *in vivo* p 50 Br J Haemat. *31* 379–385 (1976)
14 Paulo, L. G., Roh, B L., and Fisher J W Antagonism of erythropoietin production in rabbits by atropine. Proc. Soc. exp. Biol. Med. *139* 207–210 (1972)
15 Rodgers, G M., Fisher G W and George, W J. Lactate stimulation of renal cortical adenylate cyclase: a mechanism for erythropoietin production following cobalt treatment of hypoxia. J Pharmac. exp. Ther *190* 542–550 (1974).
16 Rodgers, G M., Fisher J W and George, W J Renal cyclic AMP accumulation and adenylate cyclase stimulation by erythropoietic agents. Am. J Physiol. *229* 1387–1392 (1975).
17 Rörth, M Ipossia, affinità dell eritrocita per l'ossigeno e produzione di eritropoietina; in Garby Anemia e iposssia, pp. 145–160 (Pensiero Scientifico, Roma 1975).
18 Schooley J C. and Mahlmann, L. J Hypoxia and the initiation of erythropoietin production. Blood Cells *1* 429–448 (1975).
19 Torrance, J., Jacobs, P., Restrepo, A., Eschbach, J Lenfant, C., and Finch, C. A. Intraerythrocytic adaptation to anemia, New Engl. J Med. *283* 165–169 (1970).

Received. September 1 1978
Accepted. November 15 1978

Dr Maria Vittoria Ambrosini,
Istituto di Biologia generale dell'Università,
Via del Giochetto, I-06100 Perugia (Italia)

the patients showed a statistically significant decrease in comparison to control values ($p < 0.01$). The urinary zinc excretion in patients with homozygous β-thalassemia was found to be higher than in controls. This increase was statistically significant ($p < 0.01$).

Discussion

It has been demonstrated that the values of the plasma zinc levels found in this study corroborates with the values reported in the literature [4, 5]. However, plasma zinc level does not correctly represent total body zinc load, particularly in the presence of hemolysis [12], since zinc is stored in a higher quantity in red blood cells than in any other tissues of the body [12]. Therefore, we have determined the zinc content of erythrocytes simultaneously with the plasma zinc level and the zinc content of erythrocytes was found to be low in patients with homozygous β-thalassemia.

On the other hand, because of its slower turnover, the zinc content of the hair reflects total body zinc more accurately [13, 14]. In fact, zinc content of the hair in patients with homozygous β-thalassemia proved to be low in our determination, as it was for erythrocytes and plasma.

These findings suggest that there is a zinc deficiency in patients with thalassemia. Besides, there was retardation of growth in all but 4 of our patients and significant hypogenitalism in addition to delayed puberty in 2 cases. These clinical signs are similar to the clinical findings in primary zinc deficiency syndrome [15–20], thereby supporting our laboratory data.

Various factors such as inadequate nourishment [21], absorption defects, geophagia [21, 22] and parasitosis [17, 21] may play a significant role in the development of zinc deficiency but none of them has been observed in our patients.

Urinary loss of zinc is another factor which may contribute to the zinc deficiency [4, 13]. Since the urinary zinc level has been found to be significantly higher in our patients than in controls, zinc deficiency may well be attributed to the urinary loss.

Cirrhotic changes due to hemochromatosis in thalassemia may be responsible for hyperzincuria as it is known that there is an abnormal urinary loss of zinc in cirrhosis [23–25]. Although we did not perform routine liver function tests, there was a significant hyperzincuria in 2 patients in which liver biopsy has revealed the findings of liver cirrhosis. An increased rate of glomerular filtration of zinc which is seen in chronic hemolysis [12] can also be responsible for hyperzincuria.

References

1 Constantoulakis, M.; Panagopoulos, G.; and Augoustaki, O.: Stature and longitudinal growth in thalassemia major. Clin. Pediat. *14*: 355–357 (1975).

2 Modell, B.: Management of thalassemia major. Br. med. Bull. *32*: 270–276 (1976).

3 Weatherall, D. J. and Clegg, J. B.: The thalassemia syndromes; 2nd ed., p. 75 (Blackwell, London 1972).

4 Arcasoy, A. and Çavdar, A. O.: Changes of trace minerals (serum iron, zinc, copper and magnesium) in thalassemia. Acta haemat. *53*: 341–346 (1975).

5 Prasad, A. S.; Diamond, M.; Gabr, M.; Sandstead, H. H.; Mokhtar, N.; and Hefny, A. E.: Biochemical studies in thalassemia. Ann. intern. Med. *62*: 87–96 (1965).

Plasma [7] erythrocyte [8], hair [9] and urinary [10] zinc levels were determined by 'Model 103 Perkin-Elmer Atomic Absorption Spectrophotometer'

The statistical evaluations were made by double variance analysis [11]

Results

The values obtained from patients and controls are shown in table I and figure 1 Plasma erythrocyte and hair zinc levels of

Table I Zinc levels in the patients and controls

	n	Plasma Zn µg/100 ml $\bar{x} \pm SD$	Erythrocyte Zn µg/ml ($\bar{x} \pm SD$)	Erythrocyte Zn µg/10^{10} erythrocytes ($\bar{x} \pm SD$)	Erythrocyte Zn µg/g/Hb ($\bar{x} \pm SD$)	Hair Zn µg/g/hair	Urine Zn µg/24 h
Controls	20	114.95 ± 22.53	18 44 ± 4 06	20.88 ± 4 77	69 63 ± 15.90	196.72 ± 48 61	315 17 ± 133 02
Patients	20	78.1 ± 22.71	12.46 ± 4 0	15.33 ± 4.56	54.95 ± 14 13	104 74 ± 36.24	712.1 ± 333.06
p		< 0 01	< 0.01	< 0.01	< 0 01	< 0.01	< 0.01

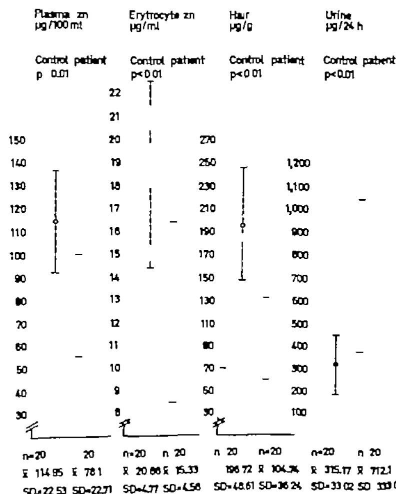


Fig. 1 Zinc levels in the patients and controls.

Acta haemat. 62: 45–50 (1979)

Composition of the γ-Chains of Human Fetal Hemoglobin at Birth and during Intrauterine Life[1]

Giuseppe Saglio, Umberto Ma..a, Clara Camaschella, Tullio Melord, Maria Ausilia Ciocca-Vasino, Pier Giorgio Pich and Giuseppe Ricco[2]

Istituto di Medicina Interna, Cattedra di Clinica Medica II, Università di Torino, Turin, and Istituto di Clinica Pediatrica, Università di Sassari, Sassari

Key Words. Hb F · γ-Chain composition · $^T\gamma$-Chain

Abstract. The composition of the γ-chains has been examined in 21 fetuses and premature babies and in 18 full-term newborns. $^A\gamma/^A\gamma$ ratio was slightly higher in fetuses (0.81 ± 0.04 expressed as glycine residue at position 136) than in full term newborns (0.73 ± 0.06), while the incidence and the percentage of $^T\gamma$-chains were clearly higher in the first group and overlap the values found in the Hb F from β-thalassemia homozygotes. On the basis of these data the authors hypothesize that $^T\gamma$-chains may be codified by a gene more active during intrauterine life than at birth. Possibly their synthesis may be reactivated when high levels of Hb F are present during adult life, as in β-thalassemia.

Introduction

The presence of either a glycine or an alanine residue at position 136 of the human γ-chains suggests that there are at least two γ-genes, one coding glycine and the other alanine at the 136th residue ($^G\gamma$ and $^A\gamma$ respectively) [15]. To explain the different $^G\gamma/^A\gamma$ ratios found at birth and during adult life and also the various percentages of the γ-chain mutants, *Huisman et al.* [8] hypothesized four structural γ-loci, two $^G\gamma$ and two $^A\gamma$ coding γ-chains in different proportions. However, the direct estimate of the number of human γ-genes supports the existence of two, or at the most three γ-genes for the human aploid genome [13]. On this basis, a new model with three γ-genes, two $^G\gamma$ (G and g) and one $^A\gamma$ has been proposed [7, 9].

In a previous paper [14] we reported the finding of a new type of γ-chain carrying threonine instead of isoleucine at position 75 ($^T\gamma$-chain): this chain was found in 29 out of 32 Italian homozygotes for β-thalassemia, in percentages up to 40% of the total γ-chains. The same chain was detected in low percentages (about 10%) in only 40% of full-term newborns. Recently *Huisman et al.* [9] reported $^T\gamma$-chains in the Hb F of a large series of cord blood samples and of

[1] This work was supported in part by grant 70.01312.04 from the 'Consiglio Nazionale delle Ricerche', Rome.

[2] We thank Dr. *L. F. Bernini* for valuable advice and technical assistance. The method employed in CM-3 purification is according to *Dr. Bernini.*

6 Atwater J and Erslev A. J., Fetal hemoglobin alkali denaturation test, in Williams, Beutler Erslev and Rundles, Hematology p 1369 (McGraw Hill, New York 1972).
7 Perkin-Elmer Clinical methods for atomic absorption spectroscopy Determination of copper in serum, AA-Cu 1 1 Determination of zinc in serum, AA Zn-1 1 (Perkin-Elmer Norwalk 1971)
8 Rosner F and Gorfien, P C. Erythrocyte and plasma zinc and magnesium levels in health and disease. J Lab clin. Med. *72* 213–219 (1968)
9 Schroeder H. A. and Nason, P A. Tracemetals in human hair J invest. Derm. *53* 71 (1969).
10 Willis, J B., Determination of lead and other heavy metals in urine by atomic absorption spectroscopy Analyt. Chem. *34* 614 (1962)
11 Düzgüneş, O İstatistik pp 113 135 229 (Ege Üniversitesi, Matbaası 1963).
12 Prasad, A. S., Schoomaker E. B., Ortega, J., Brewen, G J., Oberleas, D and Oelshlegel, F., jr Zinc deficiency in sickle cell disease Clin. Chem. *21* 582–587 (1975)
13 McBean, L. D Mahloudji, M. S., Reinhold, J G and Halsted, J A. Correlation of zinc concentrations in human plasma and hair Am. J clin. Nutr *24* 506–509 (1971).
14 Valkovic, V., Miljanic, D., Wheeler R. M Liebert, R. B., Zabel, T and Phillips, G C., Cariation in trace metal concentrations along single hairs as measured by proton-induced X-ray remission photometry Nature, Lond. *243* 543–544 (1973).
15 Burch, R. E. Hahn, H. K. J and Sullivan, J F., Newer aspects of the roles of zinc, manganese and copper in human nutrition. Clin. Chem. *21* 501 (1975)
16 Coble, D Y jr Bardin, C. W., Ross, G T and Darby W T Studies of endocrine function in boys with retarded growth, delayed sexual maturation and zinc deficiency J clin. Endocr Metab. *32* 361–367 (1971).
17 Prasad, A. S. Miale, A., jr., Farid, Z., Sandstead, H. H., and Schulert, A. R.. Zinc metabolism in patients with the syndrome of iron deficiency anemia, hepatosplenomegaly dwarfism and hypogonadism. J Lab. clin. Med. *61.* 537–549 (1963).
18 Burch, R. E. and Sullivan, J F Diagnosis of zinc, copper and manganese abnormalities in man. Med. Clins N Am. *60* 655–660 (1976).
19 Henkin, R. L. Trace metals in endocrinology Med. Clins N Am. *60* 779–793 (1976).
20 Sandstead, H H., Prasad, A. S., Schulert, R. R. Farit, Z. Miale, A., Basilly S., and Darby W J., Human zinc deficiency Endocrine manifestations and response to treatment. Am. J. clin. Nutr *20* 422–442 (1967).
21 Orten, J M.. Biochemical aspect of zinc metabolism in Prasad, *Zinc* metabolism, p. 38 *(Thomas, Springfield 1966)*
22 Mikac Devic, D.. Methodology of zinc determinations and the role of zinc in biochemical processes. Adv clin. Chem. *13* 271 (1970).
23 Prasad, A. S. Oberleas, D and Halsted, J A. Determination of zinc in biological fluids by atomic absorption spectrophotometry in normal and cirrhotic subjects. J Lab. clin. Med. *66* 508–516 (1965).
24 Singh, D Vijavargia, R., Dabke, A. T., Khan, M. A., Chhaparwal, B C., and Matur P S. Serum zinc estimation in hepatic disorders of infancy and childhood. Ind. Pediat. *9* 593–598 (1972).
25 Walker B. E. Dawson, J B., Kelleher J and Losowsky M. S. Plasma and urinary zinc in patients with malabsorption syndromes or hepatic cirrhosis. Gut *14* 943–948 (1973).

Received. June 6, 1978
Accepted. December 1 1978

Ülker Doğru, Hematology Division of
Department of Pediatrics,
Faculty of Medicine, University of Ankara,
Ankara (Turkey)

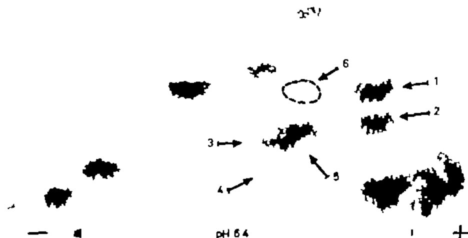


Fig. 1. Tryptic fingerprint on paper of non-aminoethylated γ-chains from premature baby N 13 showing high levels of ᵀγ-chains. 1 = Peptide Tp9a (residues 67–76) with isoleucine at position 75. 2 = peptide Tp9b with threonine at position 75. 3 = peptide Tp8,9 (residues 66–76) with isoleucine at position 75. 4 = peptide Tp8/9 with threonine at position 75. 5 = peptide Tp15 (residues 133–144) completely oxidized. 6 = normal position of Tp15 (dotted area).

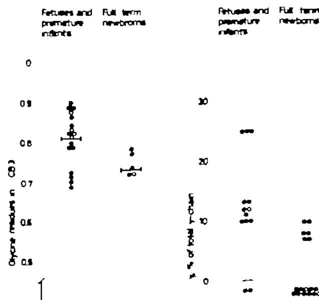


Fig. 2. Left: ᴳγ-Levels (as ᴳγ-residues in the CB-3 fragment) found in fetuses and premature babies, compared to ᴳγ-levels in full-term newborns. Right: Tγ-V lnes (as percentages of the total γ-chains) found in premature infants and fetuses compared to those found in full-term newborns

adults with several abnormal hematological conditions with a variable incidence in different racial groups

From all these data the evidence is that $^{T}\gamma$-chains are the product of a new γ-structural gene with a wide distribution coding threonine at position 75 ($^{T}\gamma$-gene) however at present the relationship between $^{T}\gamma$-gene and the models previously hypothesized for γ-genes is not completely clear

In this paper we examine the Hb F composition of 21 fetuses and premature babies, aged between 12 and 32 weeks, compared with the data obtained from 18 full-term newborns

Materials and Methods

Collection of blood samples and preparation of hemolysates were carried out by the usual procedures. Hemoglobin electrophoresis and quantitation of hemoglobin fractions were performed on cellulose acetate, as described by *Marengo-Rowe* [11] Hb F was determined by the alkali denaturation method, according to *Betke et al.* [2], modified by *Colombo et al* [4] The alkali denaturation technique was also employed in obtaining consistent aliquots of Hb F from hemolysates. Contaminating nonheme proteins, particularly carbonic anhydrase, were separated from the alkali-resistent hemoglobin by column chromatography on CM Sephadex, as described by *Bernini et al* [1] Hb F was converted into globin by the acid acetone precipitation method, and α- and γ-chains were separated on CM-cellulose, according to *Clegg et al.* [3] After tryptic digestion of the whole globin F and/or the free γ-chains, peptide maps were obtained at pH 6.4 as described by *Sick et al.* [16] (fig. 1) The $^{T}\gamma$-chain percentage was calculated by eluting, in methanol, the peptides Tp9a (75 Ileu) and Tp9b (75 Th) – previously stained with cadmium ninhydrin reagent as described by *Dreyer and Bynum* [6] – and by reading the eluates spectrophotometrically at 500 nm The peptides meant to be analyzed were stained with a solution containing 2 mg Fluram (Hoffmann-La Roche) in 100 ml of 0.1 / pyridine in acetone. After identification under UV light, the peptides were eluted with 5% acetic acid, vacuum dried and repurified by ascending chromatography on paper in a solvent composed of 98% formic acid, secondary buthanol and water (30 140 30 v/v) [14] The γ 136 Gly/Ala ratio was determined after cleavage of 10 mg whole globin F with 100-fold molar excess of CN Br in 70% formic acid at room temperature for 24 h. CNBr was eliminated by vacuum drying, and the residue was fingerprinted on paper at pH 6.4 In order to separate the γCB-3 fragment, as described by *Bernini et al.* [1] and by *Kamu ora et al.* [10] Amino acid analysis was performed on an LKB model 4101 amino acid analyzer after elution of the peptides directly from the paper with 6 *N* HCl and their hydrolysis *in vacuo* at 110 °C for 24 h.

Results

All the cases have been tested for the presence of the $^{T}\gamma$-chains, while $^{G}\gamma/^{A}\gamma$ ratios have been determined in 17 fetuses and premature babies and in 10 newborns. The results have been summarized in table I The $^{G}\gamma/^{A}\gamma$ ratio has been expressed as the number of glycine and alanine residues present in the CB 3 fragment. $^{T}\gamma$-Chains have been expressed as percentage of the total γ-chains.

Glycine residues of fetuses and premature infants range from 0 70 to 0.90 with a mean value of 0 81 ± 0 04 while in full-term newborns they range from 0 66 to 0.81 with a mean value of 0 73 ± 0 06 as shown in figure 2. The data observed in Italian full-term newborns overlap those reported in the literature [8] while glycine residues of fetuses and premature babies of our series are slightly higher than the corresponding values of *Nute et al* [12]

$^{T}\gamma$-Chains have been found in 19 out of 21 cases during intrauterine life, in amounts of 8–33 / of the total γ-chains on the contrary only 7 out of 18 newborns present low

Table I (continued)

Case No.	Gestational age, weeks	Hb F %	$^{T}\gamma$% of the total Hb F	γCB-3	
				Gly	Ala
Full-term newborns					
18		75	absent	0.78	2.27
Mean values				0.73	2.29

ND = Not determined.
The method of Betke was modified according to *Colombo et al.* [4]
The subject was transfused before examination
In cases 3, 7, 13 and 14, a second determination of $^{T}\gamma$-chain percentage gave the following values: 8, 11, 13 and 9%, respectively

percentages of $^{T}\gamma$-chains (about 10%), as shown in figure 2.

Discussion

$^{T}\gamma$-Chains have been detected in the Hb F of 29 out of 32 Italian homozygotes for β-thalassemia in percentages variable from traces to 40% [14]. In the series of *Huisman et al.* [9] $^{T}\gamma$-chains have been found in 7 out of 10 β-thalassemia homozygotes from Italy and Greece, in amounts ranging from 21 to 47% of the total γ-chains. On the other hand, the Hb F from cord blood samples contains lower amounts of $^{T}\gamma$-chains: in fact, in our series only 7 out of 18 newborns present about 10% of $^{T}\gamma$-chains, and *Huisman et al.* have reported $^{T}\gamma$-chains in 28 out of 98 cord blood samples in a percentage of about 20%. We cannot exclude that the fact that the difference observed between $^{T}\gamma$-chain percentage in the two series may be attributed to the different techniques employed [9, 14].

However, in our cases, during intrauterine life the incidence and the levels of $^{T}\gamma$-chains are clearly higher than in the newborns and are similar to those found in β-thalassemia. Thus, $^{T}\gamma$-chains seem to be the product of a γ-gene mostly active during prenatal life and partially or completely suppressed at birth. Possibly this gene can be reactivated when high levels of Hb F are present during adult life, as in β-thalassemia or in some related conditions [9, 14].

On the other hand during intrauterine life the $^{T}\gamma$-chain percentage varies widely and in 2 cases they seem to be completely absent; furthermore, the $^{T}\gamma$-chain amount is not related to gestational age. Whether the $^{T}\gamma$-chain percentage variability and sometimes the absence are due to a variable expression of the $^{T}\gamma$-gene or to the presence of different γ-genotypes (with and without the $^{T}\gamma$-gene) cannot, at present, be easily assessed.

However our knowledge of the $^{T}\gamma$-gene is still inadequate to allow any new statements about the γ-gene number and expression. In fact, the nature of the residue at position 136 of the $^{T}\gamma$-chain is still under discussion: while previous results had suggested the presence of glycine in this position

Table I

Case No	Gestational age weeks	Hb F %[1]	$^{T}\gamma$% of the total Hb F	γCB-3 Gly	γCB-3 Al
Fetuses and premature infants					
1	12	ND	12	0.71	2.26
2	14	ND	10	ND	
3	16	90	30	0.89	2.22
4	18	87	13	0.88	-21
5	18	92	10	0.90	2.15
6	20	86	15	0.83	2.26
7	20	90	18	0.81	2.18
8	20	89	13	0.78	2.21
9	24	92	22	0.70	2.35
10	28	88	20	ND	
11	28	90	11	0.79	2.14
12	28	84	absent	0.89	2.22
13	30	89	33	0.84	2.19
14	30	85	25	0.87	2.15
15	32	89	25	ND	
16	32	83	25	ND	
17	32	80	8	0.83	2.10
18	32	83	12	0.79	2.25
19	32	60[2]	absent	0.82	2.27
20	32	88	18	0.72	2.29
21	32	85	10	0.69	2.33
Mean values				0.81	2.22
Full-term newborns					
1		76	absent	ND	
2		90	7	0.72	2.40
3		83	10[3]	ND	
4		80	absent	ND	
5		87	absent	ND	
6		89	8	0.70	2.28
7		85	8[3]	ND	
8		91	absent	ND	
9		72	7	0.77	2.25
10		86	absent	ND	
11		84	bsent	0.66	2.31
12		93	absent	0.72	2.35
13		81	10[3]	0.69	2.27
14		78	6	0.73	2.30
15		70	absent	ND	
16		70	absent	0.81	2.17
17		81	absent	0.75	2.26

Short Communication

Acta haemat. 62: 51–53 (1979)

Effect of a Constant Non Uniform Magnetic Field on Leucocyte Migration *in vitro*

Preliminary Study

A. Bellossi and J. de Certaines

Laboratoire de biophysique, UER Clinique et thérapeutique médicale, Rennes

Key Words. Non-uniform constant magnetic field · Leucocyte migration

Abstract. The migration *in vitro* of circulating blood leucocytes was observed in a non-uniform constant magnetic field. The migration surface was stretched towards zones of high induction. The hypothesis of an orientation of the membrane molecule and particularly of the lipid constituents emerged.

An action *in vitro* of magnetic fields on leucocytes has been shown by previous experiments [1–4, 6, 10, 11]. We here present the first conspicuous effect of a constant non-uniform magnetic field on leucocyte migration *in vitro*.

Material and Method

20 cm³ of blood were collated on 5 drops of heparin from veins at the fold of the elbow using presumably healthy donors chosen at random. After sedimentation (3–4 h) at an ambient temperature, the plasma containing the leucocytes was set apart. The supernatant liquid was centrifuged for 10 min at 1,000 rpm. The plasma was eliminated, the sediment washed three times in TC 199 (Difco), then mixed with 2 cm³ of TC 199. The resulting solution was divided between micrometric blood-measuring tubes by capillarity. The tubes were then sealed with Seal-ease. They were centrifuged for 5 min at 2,000 rpm and were then cut horizontally at the sediment and supernatant interface. The fragment containing the suspension was fixed on the lateral facet of an Altuglas cuvette. The cuvette had an outer diameter of 2.64 cm, and inner diameter of 2.04 cm, a height of 0.55 cm and a volume of 1 cm³. The fixation was effected by a spot of silicon grease. The cuvette was filled with TC 199. A slide prevents evaporation.

Eight of these cuvettes were placed in the gap of an electromagnet. One of its poles was flat and had a diameter of 5 cm. A conical ferromagnet, diameter 3 cm and height 2 cm, was applied to the second pole to obtain a heterogeneous magnetic field. The principal component of the field was horizontal. The field strength at the top of the conical ferromagnet was 8,000 OE with a gradient of about 1,700 OE/cm (Hall-type gaussmeter). Migration took place between 15 and 17 h in the field, the conical pole being either northern or southern, depending on the samples.

We thank Dr. Genetet, Director of the Centre de transfusion sanguine, Rennes, for having facilitated the acquisition of blood samples.

[14] preliminary results recently obtained on γ-chains separated by isoelectric focusing, seem to indicate that the $^{T}\gamma$-chain may be an $^{A}\gamma$-variant [5]

References

1 Bernini, L. F., Fiorelli, G. Matuonto, V., Bianchi, G. e Chiesa, G. Polimorfismo strutturale dell emoglobina umana. *Atti riunioni* gruppo per lo studio dell'eritrocita, p 25 (Cilag, Milano 1971)

2 Betke, K. Marti, H. R., and Schlicht, I. Estimation of small percentages of fetal hemoglobin. Nature, Lond. *184* 1877–1879 (1959).

3 Clegg, J B Naughton, W A and Weatherall D J Abnormal human hemoglobin. Separation and characterization of the alpha and beta chains by chromatography and the determination of two new variants, Hb Chesapeake and Hb J Bangkok. J molec. Biol. *19* 91–108 (1966)

4 Colombo, B., Kim B Perez Atencio, R., Molina, C., and Terrenato, L. The pattern of fetal hemoglobin disappearance after birth Br J Haemat. *32* 79–87 (1976)

5 Comi, P., Giglioni B Ottolenghi, S., Gianni A. M. Ricco G Mazza U., Saglio, G Camaschella, C. Pich, P G., Gianazza, E., and Righetti, P G $^{G}\gamma$ and $^{A}\gamma$ globin chains *separation and quantitation by isoelectric focusing.* Biochem. biophys. Res. Commun (in press)

6 Dreyer W J and Bynum, E. In Colowick and Kaplan, Methods in enzymology vol 11 pp 32–39 (Academic Press, New York 1967)

7 Huisman, T H J., Harris, H. Gravely M Schroeder W A. Shelton, J R. Shelton, J B., and Evans, L. The chemical heterogeneity of the fetal hemoglobin in normal newborn infants and adults. Mol. cell. Biochem. *17* 45–55 (1977)

8 Huisman, T H J., Schroeder W A. Bannister W H and Grech, J L. Evidence for four non allelic structural genes for the γ chain of human fetal hemoglobin. Biochem. Genet. *7* 131–139 (1972)

9 Huisman, T H J Schroeder W A Reese, A. Wilson, J B., Lan, H Shelton, J R., Shelton, J B., and Baker S. The $^{T}\gamma$-chain of human fetal hemoglobin at birth and in several abnormal hematological conditions. Pediat. Res. *11* 1102–1105 (1977).

10 Kamuzora, H Ringelhann, B Konotey-Ahulu, F I D Lehmann, H and Lorkin, P A. Further investigations on the chain of a Ghanian adult, homozygous for hereditary persistence of foetal hemoglobin. Isolation of γCB-3 fragment and determination of $^{G}\gamma$-^{A} ratio in human haemoglobins. Acta haemat. *53* 315–320 (1975).

11 Marengo-Rowe, A. J. Rapid electrophoresis and quantitation of hemoglobins on cellulose acetate. J clin. Path. *18*. 790–795 (1965).

12 Nute, P E., Pataryas, H. A., and Stamatoyannopoulos, G. The $^{G}\gamma$ and $^{A}\gamma$ hemoglobin chains during human fetal development. Am J hum. Genet. *25* 271–276 (1973).

13 Old, J Clegg, J B., Weatherall, D J Ottolenghi, S., Comi, P., Giglioni, B Mitchel, J Tolstoshev P and Williamson, R.. A direct estimate of the number of human γ globin genes. Cell *8* 13–18 (1976).

14 Ricco, G Mazza, U., Turi, R. M Pich, P G Camaschella, C. Saglio, G and Bernini, L. F Significance of a new type of human foetal haemoglobin carrying a replacement isoleucine → threonine at position 75 (E19) of the γ chain. Hum. Genet. *32* 305–313 (1976).

15 Schroeder W A., Huisman, T H J Shelton, J R. Kleihauer E. F Dozy A. M., and Roberson, B Evidence for multiple structural genes for the γ chain of human fetal hemoglobin. Proc. natn. Acad. Sci. USA *60* 537 544 (1968)

16 Sick, K. Beale, D Irvine, D Lehmann, H Goodall, P T and McDougall, S. Hemoglobin G Copenhagen and hemoglobin J Cambridge. Two new β chain variants of hemoglobin A. Biochim. biophys. Acta *140* 231–242 (1967).

Received. October 16, 1978
Accepted December 4 1978

Dr G Saglio, Istituto di Clinica Medica II,
Università di Torino, via Genova 3,
10126, Turin (Italy)

Short Communication

Acta haemat. 62: 51–53 (1979)

Effect of a Constant Non-Uniform Magnetic Field on Leucocyte Migration *in vitro*

Preliminary Study

A. Belloasi and J. de Certaines

Laboratoire de biophysique, UER Clinique et thérapeutique médicale, Rennes

Key Words. Non-uniform constant magnetic field · Leucocyte migration

Abstract. The migration *in vitro* of circulating blood leucocytes was observed in a non-uniform constant magnetic field. The migration surface was stretched towards zones of high induction. The hypothesis of an orientation of the membrane molecule and particularly of the lipid constituents emerged.

An action *in vitro* of magnetic fields on leucocytes has been shown by previous experiments [1–4, 6, 10, 11]. We here present the first conspicuous effect of a constant non-uniform magnetic field on leucocyte migration *in vitro*.

Material and Method

20 cm^3 of blood were collated on 5 drops of heparin from vein in the fold of the elbow using presumably healthy donors chosen at random. After sedimentation (3–4 h) at an ambient temperature, the plasma containing the leucocytes was set apart. The supernatant liquid was centrifuged for 10 min at 2,000 rpm. The plasma was eliminated, the sediment washed three times in TC 199 (Difco), then mixed with 2 cm of TC 199. The resulting solution was divided between micrometric blood-measuring tubes by capillarity. The tubes were then sealed with Seal-ease. They were centrifuged for 5 min at 2,000 rpm and were then cut horizontally at the sediment and supernatant interface. The fragment containing the suspension was fixed on the lateral facet of an Altuglas cuvette. The cuvette had an outer diameter of 2.64 cm, and inner diameter of 2.04 cm, height of 0.55 cm and volume of 1 cm. The fixation was effected by spot of silicon grease. The cuvette was filled with TC 199. A slide prevents evaporation.

Eight of these cuvettes were placed in the gap of an electromagnet. One of its poles was flat and had diameter of 5 cm. A conical ferromagnet, diameter 3 cm and height 2 cm, was applied to the second pole to obtain heterogeneous magnetic field. The principal component of the field was horizontal. The field strength at the top of the conical ferromagnet was 8,000 OE with gradient of about 1,700 OE/cm (Hall-type gaussmeter). Migration took place between 15 and 17 h in the field, the conical pole being either northern or southern, depending on the samples.

W thank Dr. *Genetet*, Director of the Centre de transfusion sanguine, Rennes, for having facilitated the acquisition of blood samples.

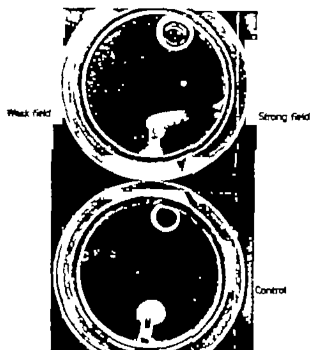


Fig. 1 Leucocyte migration. Upper photo: migration in a non uniform constant magnetic field. Lower photo: migration out of a magnetic field.

Result and Comment

The migration surface was deformed. It stretched towards the nearest pole, hence towards the part of the field where induction was highest (fig. 1). Whether this pole was northern or southern was of no importance.

Such migrations show the existence and efficiency of a magnetic force and a paramagnetic character of the leucocytes. However this force does not appear immediately. First the cells leave the capillary tube then they are subjected to the magnetic induction. So when the direction of the tube is the same as the direction from one pole to the other and the tube exit faces the flat pole the cells leave the tube and go to the conical pole. The erythrocytes can follow the leucocytes: the first motion is very likely due to the leucocytes but at this stage of the experiment it does not seem possible to say whether leucocytes are actively or passively deviated.

This appearance of the paramagnetic nature in a new stable structure implies that the effect of a molecular orientation lasts a sufficient length of time, the cell behaving like a Kelvin body.

Only sufficiently large molecules can be oriented by a magnetic field [5, 9]. Many molecules are present in the liquid crystal form in living organisms.
They are therefore able to orient themselves uniformly [12]. This is so for membrane lipids. At the membrane level, lipids and proteins do not contract covalent bonds. Membrane stability is due to the interaction of low energy. A structure modification would only require an extremely small energy consumption [8] and the magnetic field would lead to a different 'membrane unit' [7].

References

1 Bellossi, A. et Bellossi, G.: Effet d'un champ magnétique uniforme sur le diamètre des lymphocytes des sujets sains et de porteurs de cancer primitif du foie. Revue CHU 24: 35–36 (1974).
2 Bellossi, A., Certaines, J. de, and Toujas, J. P.: Effect of constant magnetic field on the reduction of nitroblue tetrazolium by the leukocytes. Wadley med. Bull. 6: 17–21 (1976).
3 Bellossi, A., Duclos, M. et Bellossi, G.: Influence d'un champ magnétique uniforme sur les leucocytes. Etude de la phagocytose. Bull. Soc. méd. Afr. noire Lang. fr. 19: 192–195 (1974).
4 Bellossi, A., Rioche, M. et Abondo, A.: Effet d'un champ magnétique uniforme sur les leucocytes. Etude de la cytotoxicité sur cellules de cancer primitif du foie. Ouest méd. 28: 365–368 (1975).
5 Dorfman, A. G.: L'action spécifique des champs magnétiques sur les macromolécules

diamagnétiques dans les solutions Biofizic URSS 7 733–734 (1962).
6 Kholodov Y K. Magnétisme en biologie Nauka, Moscow 1970).
7 Lehninger A. Bases moléculaires de la structure et des fonctions cellulaires, dans Biochimie; 6e éd., pp. 212, 398, 405 (Flammarion, Paris 1972).
8 Mazliak, P Les membranes protoplasmiques (Doein, Paris 1971).
9 Piruzian, L. A., Gleser W M Dementev V A., Lomonosov V A. et Tchibrikin, W M Mécanismes biologiques d'action des champs magnétiques permanents. Akad. Nauk SSSR, Ser biol. 4 535–539 (1970).
10 Toma, L., Oprean, R., Cordea, L et Gavrila A. L. Contribution à l'action stimulative d'un champ magnétique constant et homogène sur l'activité phagocytaire. Rev roum. Physiol. 3 133–140 (1966).
11 Topala, N. and Ailenei, O Some aspects concerning the influence of electromagnetic fields on immunological reactions. The influence of homogeneous magnetic fields on phagocytosis in vitro Rev roum. Biol. Zool. 14 443–447 (1969).
12 Wyart, J Cours conférence No. 33 (Sopodos, Paris, 1937638 CPT).

Received, July 25 1978
Accepted. December 9, 1978

A Belloni, Laboratoire de biophysique,
UER Clinique et thérapeutique médicale,
F-35043 Rennes Cedex (France)

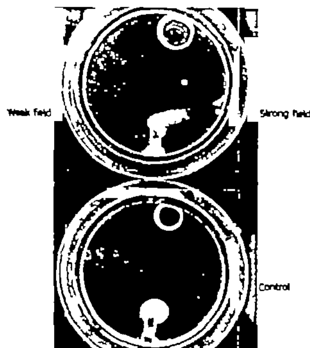


Fig. 1 Leucocyte migration. Upper photo migration in a non uniform constant magnetic field. Lower photo migration out of a magnetic field.

Result and Comment

The migration surface was deformed. It stretched towards the nearest pole, hence towards the part of the field where induction was highest (fig. 1) Whether this pole was northern or southern was of no importance

Such migrations show the existence and efficiency of a magnetic force and a paramagnetic character of the leucocytes. However this force does not appear immediately First the cells leave the capillary tube then they are subjected to the magnetic induction. So when the direction of the tube is the same as the direction from one pole to the other and the tube exit faces the flat pole the cells leave the tube and go to the conical pole The erythrocytes can follow the leucocytes the first motion is very likely due to the leucocytes but at this stage of the experiment it does not seem possible to say whether leucocytes are actively or passively deviated

This appearance of the paramagnetic nature in a new stable structure implies that the effect of a molecular orientation lasts a sufficient length of time the cell behaving like a Kelvin body

Only sufficiently large molecules can be oriented by a magnetic field [5 9] Many molecules are present in the liquid crystal form in living organisms.
They are therefore able to orient themselves uniformly [12] This is so for membrane lipids At the membrane level, lipids and proteins do not contract covalent bonds. Membrane stability is due to the interaction of low energy A structure modification would only require an extremely small energy consumption [8] and the magnetic field would lead to a different membrane unit [7]

References

1 Bellossi, A. et Bellossi, G Effet d'un champ magnétique uniforme sur le diamètre des lymphocytes des sujets sains et de porteurs de cancer primitif du foie. Revue CHU *24* 35–36 (1974)

2 Bellossi, A. Certaines, J de, and Toujas, J P Effect of constant magnetic field on the reduction of nitroblue tetrazolium by the leukocytes. Wadley med. Bull. *6.* 17–21 (1976)

3 Bellossi, A. Duclos, M et Bellossi, G Influence d un champ magnétique uniforme sur les leucocytes. Etude de la phagocytose. Bull Soc. méd. Afr noire Lang. fr *19* 192–195 (1974).

4 Bellossi, A Rioche, M et Abondo, A. Effet d'un champ magnétique uniforme sur les leucocytes. Etude de la cytotoxicité sur cellules de cancer primitif du foie Ouest méd. *28* 365–368 (1975).

5 Dorfman, A. G L action spécifique des champs magnétiques sur les macromolécules

tion of erythrocytic G-6PD deficien these 500 subjects were found to h deficiency giving an overall inci 2.2%, while sexwise it was 2.06 males and 2.32 / in the females. Th ence in the incidence between the t was found to be statistically insignific.

References

1 Dameson, S. B. and M Martin, R. B Parn. haemoglobinuria. Q Jl Med *15* 25–46 (194
2 Jolly J G Sarup, B. M Bhatnagar D and Maini, S C. Glucose-6-phosphate deh drogenase deficiency in India. J Indian med Ass. *58*. 196–200 (1972)
3 Kate, S. L., Phadke, M A., Sarnaik, G. S and G S Study of erythrocytic glu- phosph dehydrogenase and abnormal h globin endogamous community – K A survey J Ass. Phy Ind. *24* 1–3 1976
4 Saha, N and B nerjee, B Incidence of ery throcy glucose-6-phosphate dehydrogenase deficiency among different ethnic groups of India Hum Hered *21* 78–82 (1971).
5 World Health Organisation Technical Report Series Annex 10 Geographical distribution of G-6PD deficiency *366* 48–53 (1967).

Received April 4 1978
Accepted January 17 1979

Dr Narottam Bhalla,
c/ Sh. Om Parkash Bhalla, Timber Market,
Batala, Dist. Gurdaspur

Correspondence

Acta haemat. 62 54–55 (1979)

Erythrocytic G-6PD Deficiency in Punjabi Sikhs

N Bhalla J Chander and H Singh

Departments of Clinical Medicine and Clinical Pathology Government Medical College and Rajendra Hospital, Patiala, Punjab (India)

To the Editor

A varying prevalence of erythrocytic G-6PD deficiency has been reported in different racial and ethnic groups of the world [5] *Dimson and McMartin* [1] found an evidence suggestive of G-6PD deficiency among the Indians as well Since then a varying incidence of this deficiency amongst different Indian population groups has been reported by various workers (table I) However the majority of these studies pertain to mixed population groups and, therefore, are not indicative of the true incidence in distinct ethnic groups. These observations also hold true for Punjab a Northern State of India and the data regarding the incidence of G-6PD deficiency amongst the Punjabi Sikhs, a distinct endogamous group in the native stock of the Punjab are scanty

We recently screened venous blood samples from 500 Punjabi Sikhs, 258 females and 242 males, by Brewer's modified methaemoglobin reduction test for the detec

Table I. Incidence of G-6PD deficiency in various Indian communities

Study	Community screened	Total number	Percentage of deficiency
Swarup and Ghosh 1962 [in ref 2]	Bengalees	82	5
Baxi et al 1963 [in ref 2]	Parsees	225	15 7
Meera Khan 1964 [in ref. 2]	Andhra	443	4 7
	tribals		3.3–13
	non tribals		1.2
DaCosta et al. 1967 [in ref 2]	Maharashtrians	662	0.9
	Marathas	390	1
Saha and Banerjee 1971 [4]	Sikhs	94	2.13
Jolly et al 1972 [2]	Punjabis	2,000	6.9
Kate et al 1976 [3]	Maharashtrian Katkaris	438	11 4
Present study	Punjabi Sikhs	500	2.2

and iron deficiency anemia do absorb iron from therapeutic oral ferrous sulphate iron preparations like normal subjects and are not resistant to oral iron therapy and do not require parenteral iron.

References

1 *Celada, A., Rudolf, H. Herreros, V.* and *Donath, A.*: Inorganic iron absorption in subjects with iron deficiency anemia, achylia gastrica and alcoholic cirrhosis using whole body counter. Acta haemat. *60*: 182-192 (1978).
2 Heinrich, H. C.: Intestinal iron absorption in man-methods of measurement, dose relationship, diagnostic and therapeutic applications, in Hallberg, Harwerth and Vannotti, Iron deficiency pp. 13-294 (Academic Press, London 1970).
3 Heinrich, H. C.: Gastric intrinsic factor and iron absorption. Lancet *II*: 1256 (1970).
4 Heinrich, H. C.: Clinical aspects of iron absorption and turnover in Kief, Bothwell, Flach, Heinrich, Jacobs and Verner Jones, Iron metabolism and its disorders, pp. 34-58 (Excerpta Medica-American Elsevier Amsterdam 1975).
5 Heinrich, H. C., Gabbe, E. E. and Kugler G.: $^{59}Fe^{2+}$ and hemoglobin-^{59}Fe absorption in human beings do not require gastric juice or intrinsic factor. Biochem. Med. *5*: 472-482 (1971).
6 Heinrich, H. C., Gabbe, E. E., Kugler G., Whang, D. H., Hausmann, K., Bartels, H., Kuss, R., Meineke, K. H., Kugler S. und Steinzer F.: $^{59}Fe^{2+}$-Resorptions-Test und diffus verteiltes Reserveeisen der Knochenmarksmakrophagen bei Magenmukosaatrophie und nach Magen-2/3-Resektion bzw. totaler Gastrektomie. Klin. Wschr. *49*: 825-835 (1971).
7 Heinrich, H. C., Gabbe, E. E., Meineke, B. und Whang, D. H.: Die empfindliche und präzise Bestimmung der Intestinalen Eisenresorption beim Menschen durch ^{59}Fe-Ganzkörperretentions-Messung in einem 4π-Grossraum-Radioaktivitäts-Detektor. Klin. Wschr. *44*: 827-833 (1966).
8 Magnusson, B. E. O.: Iron absorption after antrectomy with gastroduodenostomy. Scand. J. Haematol., suppl. 26, pp. 1-111 (1976).

H. C. Heinrich,
Division of Medical Biochemistry
University Hospital Hamburg-Eppendorf,
Martinistrasse 52, D-2000 Hamburg 20 (FRG)

This letter was presented to Dr. *Celada*, who offered the following reply.

Absorption of inorganic iron in elemental form or combined with food is impaired in patients with achylia gastrica [1-3]. Hydrochloric acid plays a part in iron absorption, since it has been shown to increase the absorption of Cl_3Fe by achlorhydric subjects [1]. Gastric juice has also been found to have a potentiating effect on the absorption of nonheme food iron [2, 3]. On the other hand, in patients with antrectomies *Magnusson* [4] has shown a significant positive correlation between gastric acid secretion (measured as the peak acid output) and absorption of a test dose of inorganic iron. The effect of gastric juice on inorganic iron absorption may be a function of hydrochloric acid [5] or other constituents [6]. Studies on the role of gastric juice in hemoglobin iron absorption are controversial. Intrinsic factor however does play a major role in complexing hemoglobin iron and serving as an intestinal transport factor for heme [7].

The difference between our results and those of Dr. *Heinrich* in the inorganic iron absorption studies in subjects with achylia gastrica, could be due to differences in the patient groups studied.

Finally *Gross et al.* [8] describe malabsorption of iron in 7 children with iron deficiency. Following intramuscular iron ad-

Letter to the Editor

Acta haemat. 62: 56–58 (1979)

No Malabsorption of Inorganic Ferrous Iron in Patients with Achylia gastrica

Celada et al. [1] recently described in this journal a considerable malabsorption of $^{59}Fe^{2+}$ for subjects with achylia gastrica. The authors have used the same test dose of 0.56 mg $^{59}Fe^{2+}$ and the technique of whole body counting of absorbed ^{59}Fe as proposed by us [7] but their results are in contradiction with published work from our [3–6] and another laboratory [8].

Neither normal gastric juice nor an intrinsic factor is required for the intestinal absorption of $^{59}Fe^{2+}$ or hemoglobin-^{59}Fe in humans [3–6]. Subjects with histamine refractive achylia gastrica and absolute intrinsic factor deficiency (pernicious anemia in remission) absorbed 27 ± 15% [3, 5, 6] and subjects with a partial gastrectomy (Billroth I and II) 32 ± 15% [6] which was identical with the absorption of 31 ± 12% as observed for the 10 µmol (= 0.56 mg) $^{59}Fe^{2+}$ test dose in normal subjects [2–6]. *Magnusson* did confirm our results since he found no indications for iron malabsorption from the 0.56 mg $^{59}Fe^{2+}$-dose in patients with Billroth II partial gastrectomy or antrectomy and gastroduodenostomy with or without vagotomy [8]. $^{59}Fe^{2+}$ malabsorption (5.1 ± 3.3%) was observed *only* in patients with a *total* gastrectomy [6].

Hemoglobin ^{59}Fe was even better absorbed (2 fold) by subjects with achylia gastrica and absolute intrinsic factor deficiency (15 ± 5.6% from a 5 mg hemoglobin-Fe dose versus 7.5 ± 2.4% in normal subjects) [3, 5] since the acidity of normal gastric juice does reduce the bioavailability of hemoglobin-iron probably by heme polymerization and precipitation [4].

A 3-fold reduction of intrinsically ^{59}Fe-labeled *meat*-(and *liver*-)iron bioavailability was, however, demonstrated for subjects with gastric mucosa atrophy or *Billroth II* partial gastrectomy [4] and confirmed with an extrinsically ^{59}Fe-labeled test meal for subjects after antrectomy or partial gastrectomy [8]. This reduced bioavailability is however caused by, e.g., meat iron *maldigestion* and not by malabsorption since it can be corrected by an *in vitro* peptic predigestion of the ^{59}Fe-labeled pork [4].

The normal absorption of ferrous iron, the doubled hemoglobin iron absorption and the considerable reduction of meat- and liver-iron favour the assumption that dietary iron maldigestion rather than malabsorption causes the development of iron deficiency in patients with achylia gastrica or partial gastrectomy [4]. Patients with achylia gastrica

and iron deficiency anemia do absorb iron from therapeutic oral ferrous sulphate iron preparations like normal subjects and are not resistant to oral iron therapy and do not require parenteral iron.

References

1 Celada, A., Rudolf, H., Herreros, V and Donath, A.: Inorganic iron absorption in subjects with iron deficiency anemia, achylia gastrica and alcoholic cirrhosis using whole body counter Acta haemat. *60*: 182 192 (1978).

2 Heinrich, H. C.: Intestinal iron absorption in man-methods of measurement, dose relationship, diagnostic and therapeutic applications, In Hallberg, Harwerth and Vannotti, Iron deficiency pp. 213–294 (Academic Press, London 1970).

3 Heinrich, H. C.: Gastric intrinsic factor and iron absorption Lancet *II*: 1256 (1970).

4 Heinrich, H. C.: Clinical aspects of iron absorption and turnover in Kief Bothwell, Finch, Heinrich, Jacobs and Verrier Jones, Iron metabolism and its disorders, pp 34–58 (Excerpta Medica-American Elsevier Amsterdam 1975).

5 Heinrich, H C., Gabbe, E. E., and Kugler G.: $^{59}Fe^{2+}$ and hemoglobin-^{59}Fe -absorption in human beings do not require gastric juice or intrinsic factor Biochem. Med. *5*: 472–482 (1971).

6 Heinrich, H. C., Gabbe, E E., Kugler G Whang, D H., Hausmann, K., Bartels, H Kuse, R Meincke, K H K gler S. und Stelzner F $^{59}Fe^{2+}$-Resorptions-Test und diffus verteiltes Reserveeisen der Knochenmarksmakrophagen bei Magenmukosaatrophie und nach Magen-2/3-Resektion bzw totaler Gastrektomie. Klin. Wschr *49*: 825–835 (1971)

7 Heinrich, H. C Gabbe, E. E Meincke, B. und Whang, D H Die empfindliche und präzise Bestimmung der intestinalen Eisenresorption beim Menschen durch ^{59}Fe-Ganzkörperretentions-Messung in einem 4 -Großraum-Radioaktivitäts-Detektor Klin Wschr *44*: 827–833 (1966).

8 Magnusson, B. E. O Iron absorption after antrectomy with gastroduodenostomy Scand. J Haematol suppl. 26, pp. 1 111 (1976).

H. C. Heinrich,
Division of Medical Biochemistry
University Hospital Hamburg-Eppendorf,
Martinistrasse 52, D–2000 Hamburg 20 (FRG)

This letter was presented to Dr *Celada*, who offered the following reply

Absorption of inorganic iron in elemental form or combined with food is impaired in patients with achylia gastrica [1–3] Hydrochloric acid plays a part in iron absorption, since it has been shown to increase the absorption of Cl_3Fe by achlorhydric subjects [1] Gastric juice has also been found to have a potentiating effect on the absorption of nonheme food iron [2, 3] On the other hand, in patients with antrectomies *Magnusson* [4] has shown a significant positive correlation between gastric acid secretion (measured as the peak acid output) and absorption of a test dose of inorganic iron. The effect of gastric juice on inorganic iron absorption may be a function of hydrochloric acid [5] or other constituents [6] Studies on the role of gastric juice in hemoglobin iron absorption are controversial Intrinsic factor however does play a major role in complexing hemoglobin iron and serving as an intestinal transport factor for heme [7]

The difference between our results and those of Dr *Heinrich* in the inorganic iron absorption studies in subjects with achylia gastrica, could be due to differences in the patient groups studied.

Finally *Gross et al.* [8] describe malabsorption of iron in 7 children with iron deficiency Following intramuscular iron ad-

Letter to the Editor

Acta haemat. 62: 56–58 (1979)

No Malabsorption of Inorganic Ferrous Iron in Patients with Achylia gastrica

Celada et al. [1] recently described in this journal a considerable malabsorption of $^{59}Fe^{2+}$ for subjects with achylia gastrica. The authors have used the same test dose of 0.56 mg $^{59}Fe^{2+}$ and the technique of whole body counting of absorbed ^{59}Fe as proposed by us [7] but their results are in contradiction with published work from our [3–6] and another laboratory [8].

Neither normal gastric juice nor an intrinsic factor is required for the intestinal absorption of $^{59}Fe^{2+}$ or hemoglobin-^{59}Fe in humans [3–6]. Subjects with histamine-refractive achylia gastrica and absolute intrinsic factor deficiency (pernicious anemia in remission) absorbed 27 ± 15% [3, 5, 6] and subjects with a partial gastrectomy (Billroth I and II) 32 ± 15% [6] which was identical with the absorption of 31 ± 12% as observed for the 10 μmol (= 0.56 mg) $^{59}Fe^{2+}$ test dose in normal subjects [2–6]. *Magnusson* did confirm our results since he found no indications for iron malabsorption from the 0.56 mg $^{59}Fe^{2+}$-dose in patients with Billroth II partial gastrectomy or antrectomy and gastroduodenostomy with or without vagotomy [8]. $^{59}Fe^{2+}$ malabsorption (5.1 ± 3.3%) was observed *only* in patients with a *total* gastrectomy [6].

Hemoglobin ^{59}Fe was even better absorbed (2-fold) by subjects with achylia gastrica and absolute intrinsic factor deficiency (15 ± 5.6% from a 5 mg hemoglobin-Fe dose versus 7.5 ± 2.4% in normal subjects) [3, 5] since the acidity of normal gastric juice does reduce the bioavailability of hemoglobin iron probably by heme-polymerization and precipitation [4].

A 3-fold reduction of intrinsically ^{59}Fe labeled *meat*-(and *liver*-)iron bioavailability was, however, demonstrated for subjects with gastric mucosa atrophy or Billroth II partial gastrectomy [4] and confirmed with an extrinsically ^{59}Fe-labeled test meal for subjects after antrectomy or partial gastrectomy [8]. This reduced bioavailability is however caused by e.g. meat iron *maldigestion* and not by malabsorption since it can be corrected by an *in vitro* peptic predigestion of the ^{59}Fe labeled pork [4].

The normal absorption of ferrous iron, the doubled hemiglobin iron absorption and the considerable reduction of meat- and liver-iron favour the assumption that dietary iron maldigestion rather than malabsorption causes the development of iron deficiency in patients with achylia gastrica or partial gastrectomy [4]. Patients with achylia gastrica

Book Reviews

R. Silber J. LoBue and A. S. Gordon (
The Year in Hematology 1978
Plenum Press, New York 1978
XVII + 529 pp., US$ 35.40
ISBN 0-306-32402-4

This book is not strictly 'panorama of the most interesting papers published in 1978 in hematology but sequence of general reviews devot d to subjects which have particularly interested the hematologists, last year study of the action of anti-cancer agents on hematopoietic progenitor cells (chiefly CFU/S and CFU/GM); review of the culture of granulocytic stem cells and its application to clinical problems (acute leukemia, chronic myeloid leukemia and blastic transformation of CML) biochemical study of the red cell membrane and lipid metabolism; an article on hemoglobin switching A–C in sheep and F A in man; paper on the mechanism of chloramphenicol- and thiophenicol-induced aplastic anemia, an original study on megacaryocyte physiology including cell separation, maturation, functional capacity paper on the mechanisms of heparin therapy – review on the regulation of cell egress from the bone marrow paper on the endocrine role of thymus, and chemical and biological characterization of thymosine review on the present 'state of art on blood cell chalones (in fact, essentially granulocytic and lymphocytic) review of cytogenetic studies in myeloproliferative and lymphoproliferative diseases and the preleukemic states, short study on the leukemia antigens, and two review on the *present concepts on the staging, chemotherapy* and radiotherapy of Hodgkin disease and non-Hodgkin's lymphoma

As all these types of books, this one includes very different papers some of them are original contributions, others are to large measure 'remake of previously published data, others are descriptive catalogues I think that every hematologist will find at least tw or three papers very interesting I any case, this book needs to be bought by every hematological library

Y Najean, Paris

E. D Thomas (ed.)
Aplastic Anaemia
Clinics in Haematology vol. 7 No. 3, 1978
Saunders, London 1978
213 pp., £8 25 (single issue)

This issue of Clinics in Haematology is not exactly text-book on aplastic anemia, but the sum of different papers devoted to some specific aspects of the disease. An extensive bibliography of each topic is given after every chapter

This book covers clinical and therapeutic fields. A long and well-documented chapter is devoted to classification and etiology in short paper the prognostic factors are reviewed, and the use of prognosis index criticized tw chapters ar devoted to recent data on immunopathology and *in vitro* bone marrow culture techniques; very interesting and original paper on paroxysmal nocturnal hemoglobinuria and aplastic anemia was written by *W F Rosse* Surprisingly tw reviews are devoted to selective neutropenias and to the platelet production defects which are not *stricto sensu* aplastic anemias (but there is no chapter on selective erythroid aplasia).

Half of the book is dedicated to therapeutical approaches of the disease *J W Adamson* gives short but excellent review on the pharmacological stimulation of bone marrow function, and *F H Gardner* his clinical experience of the use of the 5 α- and 5 β-androstanes. Tw chapters are devoted to bone marrow graft and one to treatment by antilymphocyte globulin. Last by the supportive measures, probably the most efficient and certainly the most necessary therapeutic procedures, are excellently described.

Even if all the aspects of the disease are not studied in this book and some chapters are only recent reevaluation of previously published data, I think that this book is excellent and should be read by every haematologist I indeed gives conflicting opinions, hich are rarely simultaneously exhibited: for instance, theory of an immune process 'against' pure defect of the stem cells, or the use of pharmacological agents 'against' bone marrow transplantation.

ministration all 7 children had a significant rise in hemoglobin concentration, similar to the patient we described

References

1 Jacobs, P Bothwell, T and Charlton, R. W.. Role of hydrochloric acid in iron absorption J appl. Physiol. *19* 187–188 (1964).
2 Cook, J D Brown, G M and Valberg, L. S.. The effect of achylia gastrica on iron absorption. J clin Invest. *43* 1185–1191 (1964)
3 Jacobs, A. and Owen, G M Effect of gastric juice on iron absorption in patients with gastric atrophy Gut *10* 488–490 (1969)
4 Magnusson, B. E. O Iron absorption after antrectomy with gastroduodenostomy Scand. J Haematol suppl 26, pp. 1–111 (1976).
5 Bezwoda, W Charlton, R., Bothwell, T Torrance, J and Mayet, F.. The importance of gastric hydrochloric acid in the absorption of nonheme food iron. J Lab clin. Med. *92* 108–116 (1978).
6 Swan, C. H J and Glass, G R. J.. Iron-binding by macromolecular components of human gastric juice. J Lab clin. Med. *81* 719–732 (1973).
7 Waxman, S. Pratt, P., and Herbert, V Malabsorption of hemoglobin iron in pernicious anemia, correction with intrinsic factor-containing substances. J clin. Invest. *47* 1819–1825 (1968)
8 Gross, S. J., Stuart, M. J Swender P T and Oski, F A.. Malabsorption of iron in children with iron deficiency J Pediat. *88* 795–799 (1976)

A. Celada,
Department of Medicine,
Cantonal Hospital
CH-1206 Geneva (Switzerland)

Original Papers

Acta haemat. 62: 61-70 (1979)

Acute Lymphatic Leukemia with Mediastinal Involvement

E. Conde, M. Gonzalez, F. Lopez, A. Inondo, M. Recio, M. A. Cuadrado and A. Zubizarreta

Departments of Haematology and Pediatric Radiology of the 'Marqués de Valdecilla' Medical Centre, Santander

Key Words. Acute lymphatic leukemia · Acute lymphatic leukemia with mediastinal involvement · T-acute lymphatic leukemia · Lymphoblastic lymphoma · Convoluted lymphocytic Lymphoma

Abstract. Mediastinal involvement was found in 11 (group A) of 43 patients affected with acute lymphatic leukemia when early thoracic roentgenograms of these patients were reviewed. Several clinicobiological characteristics of these patients were compared with those with a normal mediastinum as shown in their roentgenograms (group B). Statistically significant differences were observed between group A and group B not only as far as age was concerned (13 years group A, 7.4 years group B) but also in the ratio males/females (10:1 group A, 10:20 group B) and with regard to the leukocyte counts (266 × 10⁹/liter group A, 20 × 10⁹/liter group B). In addition, patients from group A showed a greater 'tumoral mass' and a more prominent extrahematological involvement (45% group A, 15% group B). In these cases 'convoluted' cells were frequently discovered and the blastic cells exhibited significantly lower scores of PAS-positivity and more marked acid phosphatase activity than those in group B. Although the rate of complete remissions (CR) obtained in both groups was similar (80% group A, 93% group B), marked differences were observed not only in the duration of CR but in the period of survival after CR as well, both factors being more prolonged in group B patients. 50% of patients with mediastinal involvement (group A) relapsed in the first 6 months of the evolution of the disease.

Introduction

The variability of the clinical manifestations, prognosis and response to chemotherapy of acute lymphoblastic leukemia (ALL) is well known. This variability has suggested that ALL can include several diseases that are similar in their morphology but different in their behavior.

Numerous attempts have been made to correlate the prognosis of ALL with other factors such as age, sex, leukocytosis in the

For these reasons, this book is worth being in good place in all the medical libraries.

Y Najean, Paris

J V Simone (ed.)
Acute Leukaemia
Clinics in Haematology vol. 7 No 2, 1978
Saunders, London 1978
VIII + 204 pp £ 8.25 (single issue)

There is perhaps no more exacting care in the medical sciences, and certainly not in clinical haematology than the total care of patients affected with acute leukaemia (AL). *J Holland* has recently made the point that owing to the gradual but unceasing progress in AL, as confronted with the relative stagnation in chronic myelogenous leukaemia, the terms acute' and chronic have lost much of their original significance and may indeed be interchangeable. However the fact remains that in certain types and strata of AL the hitherto elusive goal of a cure is indeed attainable, therefore obliging clinical haematologists to continuously update their knowledge both of treatment and disease.

This slender volume of little more than 200 pages, carefully organized by *J V Simone* is perfectly adequate to its objective. A group of authoritative specialists give a synthetic and personalized overview on ten different sectors of the multifaceted problem of AL. *S L Georg* has written an uncommonly informative and useful chapter dealing with the design and evaluation of leukaemia trials. *A M Mauer and P H Wiernik* discuss the state of the art in the treatment of AL in children and in adults, respectively *P Alexander and R Powles* deal once again, with great clarity with the disconcerting problem of immunotherapy· their chapter should be confronted with the 1978 Symposium on Immunotherapy of Cancer edited by *W D Terry and D Windhorst* The possibilities and limits of bone marrow transplantation re present ed by the wellknown Seattle authorities, *J E. Sanders and E D Thomas* The growing importance of extramedullary leukaemia is highlighted by *H O Hustu and R. J Aur* while blood component therapy is updated by *T A Lister and R. A Yankee* Finally three fundamental basic aspects are reviewed, that is cell kinetics by *Z. A Azlia et al* human leukaemia-associated antigens by *T Mohanakumar and R. B Raney* Jr and cytogenetics by *J D Rowley*

A. M. Marmont, Genova

W J Williams E. Beutler A J Erslev and R. W Rundles
Hematology 2nd ed.
McGraw-Hill, Düsseldorf 1977
XXIV + 1755 pp DM 120.40
ISBN 0-07-070376-0

Wer die erste Ausgabe des Williams kennt, hat mit Spannung auf die Neuausgabe dieses vorzüglichen hämatologischen Lehrbuches gewartet. Das Buch wurde von den zahlreichen Mitarbeitern auf den neuesten Stand des Wissens gebracht. Das gesamte Gebiet der Hämatologie wurde in 169 übersichtlich gegliederte und gut aufeinander abgestimmte Kapitel aufgeteilt. Es liegt in der Natur der medizinischen Entwicklung, dass pathophysiologische Aspekte und Laborbefunde der einzelnen Krankheitsbilder breiten Raum einnehmen, ohne dass jedoch die Klinik zu kurz kommt. Der an speziellen Fragen interessierte Leser findet am Ende jedes Kapitels ein umfangreiches Literaturverzeichnis mit den Titeln der einschlägigen Arbeiten. In einem fast 100seitigen Anhang werden zahlreiche hämatologische Laboruntersuchungen besprochen, die weit über den Rahmen des in der Praxis üblichen Untersuchungsprogrammes hinausgehen. Obwohl der Umfang des Buches auf 1700 Seiten zugenommen hat, ist es dank der Verwendung von Dünndruckpapier nicht unhandlicher geworden.

Das Werk ist für den Hämatologen ei e unerlässliche Hilfe und eine reichhaltige Fundgrube· ferner wird es für einen grösseren Kreis hämatologisch interessierter Internisten von Wert sein und sicher Eingang in zahlreiche Bibliotheken internistischer Kliniken finden. Der Preis der 2. Auflage ist ausgesprochen niedrig.

R. Kappeler Bern

Original Papers

Acta haemat. 62. 61 70 (1979)

Acute Lymphatic Leukemia with Mediastinal Involvement

E. Conde M Gonzalez, F Lopez, A Iriondo M Recio M A Cuadrado and A Zubizarreta

Departments of Haematology and Pediatric Radiology of the 'Marqués de Valdecilla' Medical Centre, Santander

Key Words. Acute lymphatic leukemia Acute lymphatic leukemia with mediastinal involvement T-acute lymphatic leukemia Lymphoblastic lymphoma Convoluted lymphocytic Lymphoma

Abstract. Mediastinal involvement was found in 11 (group A) of 43 patients affected with acute lymphatic leukemia when early thoracic roentgenograms of these patients were reviewed. Several clinicobiological characteristics of these patients were compared with those with a normal mediastinum as shown in their roentgenograms (group B). Statistically significant differences were observed between group A and group B not only as far as age was concerned (13 years group A, 7·4 years group B) but also in the ratio males/females (10·1 group A, 10·20 group B) and with regard to the leukocyte counts (266×10^9/liter group A, 20×10^9/liter group B). In addition, patients from group A showed a greater 'tumoral mass' and a more prominent extrahematological involvement (45% group A, 15% group B). In these cases 'convoluted' cells were frequently discovered and the blastic cells exhibited significantly lower scores of PAS-positivity and more marked acid phosphatase activity than those in group B Although the rate of complete remissions (CR) obtained in both groups was similar (80% group A, 93% group B), marked differences were observed not only in the duration of CR but in the period of survival after CR as well, both factors being more prolonged in group B patients. 50% of patients with mediastinal involvement (group A) relapsed in the first 6 months of the evolution of the disease.

Introduction

The variability of the clinical manifestations, prognosis and response to chemotherapy of acute lymphoblastic leukemia (ALL) is well known. This variability has suggested that ALL can include several diseases that are similar in their morphology but different in their behavior

Numerous attempts have been made to correlate the prognosis of ALL with other factors such as, age, sex, leukocytosis in the

initial examination lymphadenopathies or hepatosplenomegaly extrahematological infiltrations and morphologic and cytochemical criteria [13 22, 26 31 32] These factors have been effective in evaluating the prognosis and their analysis together with the association of new cytochemical and immunological procedures has permitted the isolation of some forms of ALL which are characterized by the following (a) higher incidence among older male children and adolescents (b) frequent mediastinal involvement (c) initial hyperleukocytosis (d) elevated paranuclear positivity to acid phosphatase (e) thymic origin of neoplastic cells (f) rapid progression of the disease with frequent extrahematological involvement, especially the CNS and (g) very bad prognosis.

Evidence based on immunological methods of three types of ALL (B T Null) suggests that such heterogenous leukemias are related to the origin of leukemic cells [3–5 11 18 30 34 36] The existence on the other hand, of mediastinal T lymphomas with a clinical course similar to the ALL T seems to indicate that the latter is more closely related to the mediastinal type of lymphoma than to the conventional or ordinary ALL [6 16 17 19–21 23 33 34 36]

The purpose of this paper is to comment on the peculiarities of 11 cases of ALL with initial mediastinal involvement (MI)

Material and Methods

43 out of 49 patients diagnosed as having ALL during the period October 1969 to August 1977 have been included in this study They had been diagnosed on the basis of the morphological and cytochemical examination of peripheral blood and bone marrow

The initial radiological studies (chest X-rays) were reviewed in all cases before any treatment was given.

The clinical findings of 41 patients were also reviewed to select the pertinent data. The patients were classified cytomorphologically in accordance with the FAB proposal (French-American-British Cooperative Group) [2] in the following way 19 L_1 6 L_2, 1 L_3, 4 unclassifiable. The remaining 11 form the theme of this study

In 34 cases the cytochemical reaction, PAS, peroxidases and nonspecific esterases were analyzed Acid phosphatase was performed in only 5 cases, and EA and EAC rosettes in 6 cases (4 with MI)

Treatment was carried out with 40 patients and was similar in all cases. Remission was induced with vincristine-prednisone plus daunorubicine from the beginning of the 3rd cycle. The three drugs were administered from the beginning to those patients with the worst prognosis A patient with nonleukemic mediastinal lymphoma was initially treated with CVP cycles but after 8 months of treatment the patient developed a leukemic lymphoma. So this patient was then included in the ALL therapeutic regimen. Meningeal prophylaxis was carried out exclusively with intrathecal chemotherapy until 1974 From then onwards cranial radiotherapy was also added.

Maintenance treatment was given with 6-mercaptopurine and methotrexate. Reinductions were performed periodically

Treatment was stopped 4 years after the diagnosis had been made 1 patient with normal mediastinal shadows was lost to follow-up after reaching complete remission (CR) and another patient with MI died on the 3rd day of hospitalization without treatment.

Results

Initial mediastinal involvement was found in 11 cases (25%) and was apparently absent in 32 cases (75% fig. 1) Table I shows the clinical findings of patients with MI These *patients ages* ranged from 4 to 39 years. The difference in the average age of each group at the time of diagnosis was



Fig. 1. Chest X-ray from case P J G. P showing 'chimney widening of superior mediastinum and left pleural effusion

Table I. Mediastinal involvement and clinical findings

Patients	Age years	Sex	Liver cm	Spleen, cm	Lymph nodes (−/+ + + +)	Extrahematological involvement
N. C. C	13	M	2.5	5	+ + + +	testicles
J-L F Q	14	M	3.5		+	
E. R. S.	39	F	8	4	−	
P J G P	11	M				
H. C. C.	12	M	3	5	+ + +	testicles
J M. C. S.	19	M	7	6	+ +	
A-P L. P	8	M	8	8	+ +	
R. G F	4	M	13.5	7	+ + +	CNS
C F A.	4	M	2	6	+	
A. G C.	10	M	4	8	+ +	
R H. P	9	M			+ + +	CNS (37 months)

statistically significant (13 and 7.41 years, $p < 0.01$ fig. 2). When the average age of group A was compared with that of patients classified as L_1 this difference was not so marked (13 and 12.52 years) whereas when compared with that of patients classified as L_1 the difference increased considerably (13 and 5.46 years fig. 2). Differences were also to be seen in the ratio of males females in group A and group B (10 1 group A, 10/70 group B $p < 0.01$).

The tumor mass appeared greater in patients with MI. Visceromegalies and/or palpable lymphadenopathies were to be seen in 90% of these patients and in only 53 of the control group. These differences, however are not significant ($0.1 < p < 0._$).

Of the 11 patients with MI 4 (36%) presented extrahematological positive findings (table I). In 2 cases these were initially located in the testicles. In the other 2 cases

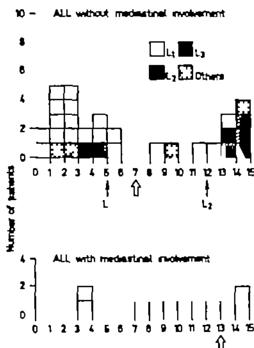


Fig. 2. Age and mediastinal involvement. White arrows point to the average age of the total of both series and black arrows point to the average age of L_1 and L_2

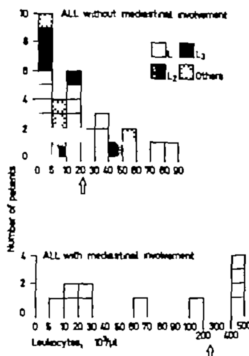


Fig. 3 Leukocytes and mediastinal involvement. The arrows point to the average leukocytosis of the two groups.

they were located in the CNS (1 case being discovered at the beginning the other 29 months after diagnoses when meninges and hypothalamus were involved, appearing as a hypotalamic obesity syndrome)

4 cases out of 30 (13%) patients without MI also presented extrahematological findings located in the CNS This discovery was made at the beginning in 2 cases (1 of these was classified L_3) 4 months later in the 3rd case and 11 months later in the 4th case.

Testicular involvement was accepted on the basis of physical examinations. 5 patients with mediastinal involvement also presented pleural effusion.

The *average number of leukocytes* in patients with MI was 266 090/µl (ranging from 7,300 to 1 000 000), whereas in those patients without mediastinal involvement the average was 20 080/µl (ranging from 2,200 to 80 800 fig. 3) It should be mentioned that a patient with mediastinal nonleukemic lymphoma was included in the first group

40% of patients with MI were found to have a significant percentage of cells with the nuclear characteristics described by *Lukes and Collins* [20] (convoluted nuclei fig 4) whereas in patients with normal mediastinal shadows the above-mentioned cells were not to be seen and the percentage of cleaved cells was much lower

Regarding *cytochemical behavior* it was noted that in patients with MI the average of positive PAS cells was 4 6% (ranging from 0 to 30%) and in those without mediastinal involvement the average was 32.5 / (ranging from 3 to 95%) These

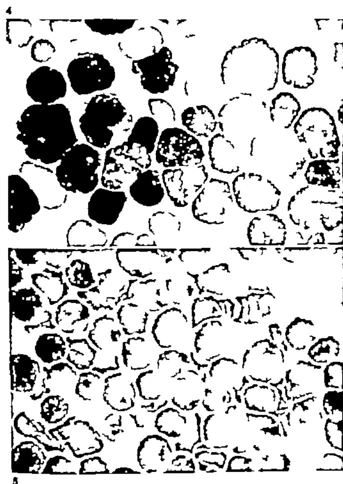


Fig. 4. Representative marrow cytology from case A-P L. P showing an infiltration by convoluted lymphoid cells and prolymphocytes

Fig. 5. Marked paranuclear acid phosphatase activity in all lymphoid cells of case J M C. S.

differences are statistically significant ($p < 0.01$).

Acid phosphatase was examined in 5 patients, 3 with MI and 2 without, the percentages of the first 3 were 85 95 and 100% (average 93.3%/ fig. 5), whereas in the second group they were 0 and 4 %. The patients with the elevated values of acid phosphatase had very low percentages of PAS-positive cells (0 0 and 10%/). On the other hand, 1 patient who had 0%/ of acid phosphatase activity had 95%/ of blastic PAS-positive cells (table II). The rest of the cytochemical reactions, peroxidases and nonspecific esterases, were negative in the ALL patients.

From the *immunological point of view* 1 of the 4 cases with MI was judged to have ALL of T cell origin because its cells formed 87%/ sheep red blood cells rosettes, most of which were stable at 37 C for 30 min.

Complete remission was achieved in 80% of the patients with MI, 2 patients failed to achieve CR and 1 was not treated.

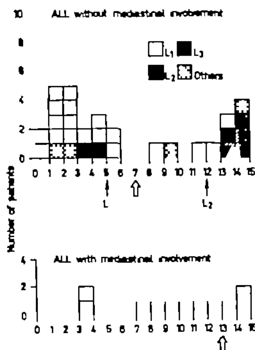


Fig. 2. Age and mediastinal involvement. White arrows point to the average age of the total of both series and black arrows point to the average age of L_1 and L_2.

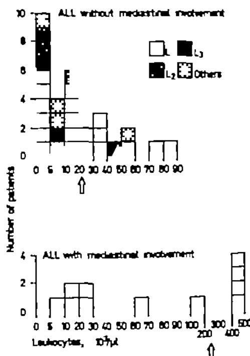


Fig. 3. Leukocytes and mediastinal involvement. The arrows point to the average leukocytosis of the two groups.

they were located in the CNS (1 case being discovered at the beginning the other 29 months after diagnoses, when meninges and hypothalamus were involved, appearing as a hypotalamic obesity syndrome)

4 cases out of 30 (13 /) patients without MI also presented extrahematological findings located in the CNS This discovery was made at the beginning in 2 cases (1 of these was classified L_2) 4 months later in the 3rd case and 11 months later in the 4th case

Testicular involvement was accepted on the basis of physical examinations. 5 patients with mediastinal involvement also presented pleural effusion

The *average number of leukocytes* in patients with MI was 266 090/μl (ranging from 7,300 to 1 000 000) whereas in those patients without mediastinal involvement the average was 20 080/μl (ranging from 2,200 to 80 800 fig. 3) It should be mentioned that a patient with mediastinal nonleukemic lymphoma was included in the first group

40% of patients with MI were found to have a significant percentage of cells with the nuclear characteristics described by *Lukes and Collins* [20] (convoluted nuclei fig. 4) whereas in patients with normal mediastinal shadows the above-mentioned cells were not to be seen and the percentage of cleaved cells was much lower

Regarding *cytochemical behavior* it was noted that in patients with MI the average of positive PAS cells was 4 6% (ranging from 0 to 30%) and in those without mediastinal involvement the average was 32.5% (ranging from 3 to 95%). These

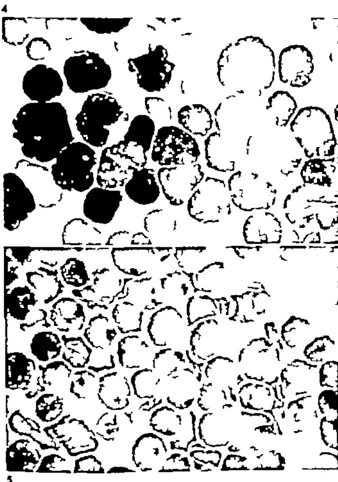


Fig. 4. Representative marrow cytology from case A-P L. P showing an infiltration by convoluted' lymphoid cells and prolymphocytes.

Fig. 5. Marked paranuclear acid phosphatase activity in all lymphoid cells of case J-M C. S

differences are statistically significant ($p < 0.01$)

Acid phosphatase was examined in 5 patients, 3 with MI and 2 without, the percentages of the first 3 were 85 95 and 100% (average 93.3%/ fig. 5) whereas in the second group they were 0 and 4 %. The patients with the elevated values of acid phosphatase had very low percentages of PAS-positive cells (0 0 and 10%). On the other hand, 1 patient who had 0% of acid phosphatase activity had 95% of blastic PAS-positive cells (table II). The rest of the cytochemical reactions, peroxidases and nonspecific esterases, were negative in the ALL patients.

From the *immunological point of view* 1 of the 4 cases with MI was judged to have ALL of T cell origin because its cells formed 87% sheep red blood cells rosettes, most of which were stable at 37 C for 30 min.

Complete remission was achieved in 80% of the patients with MI, 2 patients failed to achieve CR and 1 was not treated.

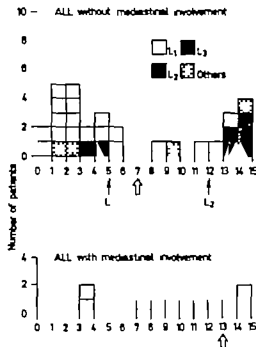


Fig. 2. Age and mediastinal involvement. White arrows point to the average age of the total of both series and black arrows point to the average age of L_1 and L_2.

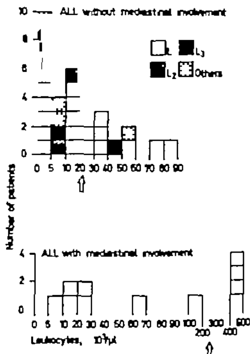


Fig. 3. Leukocytes and mediastinal involvement. The arrows point to the average leukocytosis of the two groups.

they were located in the CNS (1 case being discovered at the beginning the other 29 months after diagnoses, when meninges and hypothalamus were involved, appearing as a hypotalamic obesity syndrome)

4 cases out of 30 (13%) patients without MI also presented extrahematological findings located in the CNS This discovery was made at the beginning in 2 cases (1 of these was classified L_2) 4 months later in the 3rd case, and 11 months later in the 4th case.

Testicular involvement was accepted on the basis of physical examinations. 5 patients with mediastinal involvement also presented pleural effusion

The *average number of leukocytes* in patients with MI was 266 090/μl (ranging from 7,300 to 1 000 000) whereas in those patients without mediastinal involvement the average was 20 080/μl (ranging from 2,200 to 80 800 fig. 3) It should be mentioned that a patient with mediastinal nonleukemic lymphoma was included in the first group

40% of patients with MI were found to have a significant percentage of cells with the nuclear characteristics described by *Lukes and Collins* [20] (convoluted nuclei' fig 4) whereas in patients with normal mediastinal shadows the above-mentioned cells were not to be seen and the percentage of cleaved cells was much lower

Regarding *cytochemical behavior* it was noted that in patients with MI the average of positive PAS cells was 4 6 % (ranging from 0 to 30%) and in those without mediastinal involvement the average was 32.5% (ranging from 3 to 95%) These

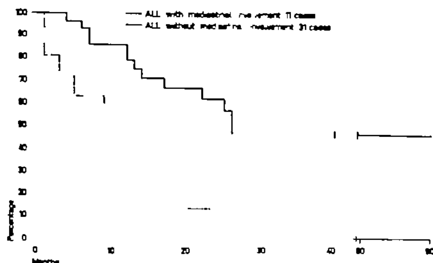


Fig. 7 Actuarial curves of survival and mediastinal involvement.

Discussion

In 1916 Sternberg described the 1st case of 'lymphoid sarcoma' and since then many cases of ALL-lymphoblastic lymphoma with MI have been described. Its exact frequency is difficult to determine due to the fact that (a) the cases studied in depth have been few (b) many of them only considered partial aspects of the process, and (c) on many occasions the authors have separated the true leukemias from the lymphoblastic lymphomas.

Taking the above facts into account, our results agree with previous papers both in incidence of mediastinal involvement and in the correlation which exists between this and the type of immunological markers in ALL (table III).

In addition, a later age at diagnosis was confirmed both in patients with mediastinal widening [10, 16, 28, 30] and in those with T-markers [1 8, 11 17 18, 25 34] with a clear predominance of males over females in both conditions.

Our findings of a more marked leukocytosis in both conditions coincided with those reported by several authors [1 5 11 14 28, 30] In approximately half of our patients (40%), a notable percentage of 'convoluted' cells were found, a fact already confirmed by the majority [14 15 24 25 35] but not by all authors [5 11] *Catovsky et al* [7 9] and later others [11 29 35] held the view that a paranuclear positivity of the alkaline phosphatase is characteristic of the T cells and, on the other hand, the PAS-positivity index of these cells is less than exhibited by the leukemic cells of the Null-LLA [7 36] Our results would indicate this to be true

Ravindranath et al [28] demonstrated that the difference between the number of survivors with and without mediastinal involvement after 12 months was statistically significant in favor of the latter Similar dif

In 5 cases (62.5%) recurrence occurred in the 14 months following CR, 2 patients died after CR due to infectious diseases and another 1 is in the initial CR at 15 months. Among the group of patients without MI CR was obtained in 28 cases (93%) and was not achieved in 2 cases. 1 patient was lost to follow up after CR had been achieved, 12 patients (42%) relapsed in the following 21

Table II. Cytochemical characteristics of 5 patients with ALL

Patients	Lymphoblasts in bone marrow %	Acid phosphatase, %	PAS %
Patients with mediastinal involvement			
1	90	85	10
2	96	95	0
3	94	100	0
Patients without mediastinal involvement			
4	97	0	95
5	36	4	5

months after CR and 15 are still in the initial CR. Figure 6 shows the actuarial duration of the first CR.

It can be observed that 50% of affected patients relapsed during the first 6 months with a maximum duration of CR of 16 months, whereas in those without MI the average duration was 21 months. The curve flattens out at about 22 months when 62% of the patients had relapsed. The maximum duration was 92 months.

The period of survival of those patients with MI is shown in figure 7 of this group 50% died during the first 9–10 months, whereas in the group without MI the patients died during the initial 25–26 months.

No patients with MI were alive after 30 months. At this point the differences with unaffected patients were statistically significant ($0.02 < p < 0.05$) The maximum period of survival of patients without MI was 93 months. It must be stressed that 4 patients who have stopped treatment belong to the group of patients not affected by MI

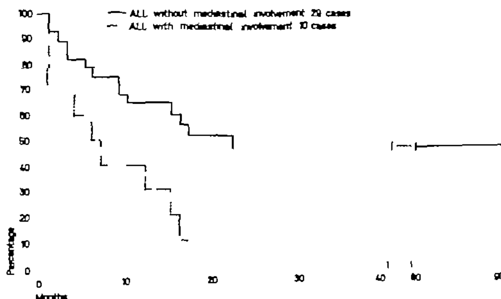


Fig. 6. Actuarial display of the first complete remission and mediastinal involvement.

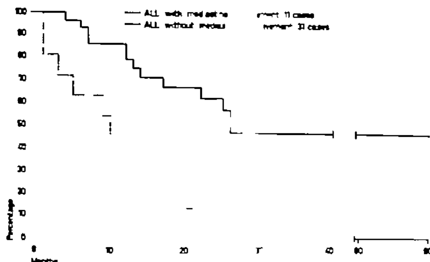


Fig. 7 Actuarial curves of survival and mediastinal involvement.

Discussion

In 1916 *Sternberg described the* 1st case of 'lymphoid sarcoma' and since then many cases of ALL-lymphoblastic lymphoma with MI have been described. Its exact frequency is difficult to determine due to the fact that (a) the cases studied in depth have been few· (b) many of them only considered partial aspects of the process, and (c) on many occasions the authors have separated the true leukemias from the lymphoblastic lymphomas.

Taking the above facts into account, our *results* agree with previous papers *both in* incidence of mediastinal involvement and in the correlation which exists between this and the type of immunological markers in ALL (table III)

In addition, a later age at diagnosis was confirmed both in patients with mediastinal widening [10, 16 28, 30] and in those with T-markers [1 8 11 17 18 .5 34] with a clear predominance of males over females in both conditions.

Our findings of a more marked leukocytosis in both conditions coincided with those reported by several authors [1 5 11 14 28 30] In approximately half of our patients (40°/), a notable percentage of 'convoluted' cells were found, a fact already confirmed by the majority [14 15 24 25 35] but not by all authors [5 11] *Catovsky et al.* [7 9] and later others [11 29 35] held the view that a paranuclear positivity of the alkaline phosphatase is characteristic of the T cells and, on the other hand, the *PAS-positivity index of these cells is less* than exhibited by the leukemic cells of the Null-LLA [7 36] Our results would indicate this to be true.

Ravindranath et al. [28] demonstrated that the difference between the number of survivors with and without mediastinal involvement after 12 months was statistically significant in favor of the latter Similar dif

In 5 cases (62.5%) recurrence occurred in the 14 months following CR, 2 patients died after CR due to infectious diseases and another 1 is in the initial CR at 15 months. Among the group of patients without MI CR was obtained in 28 cases (93%) and was not achieved in 2 cases 1 patient was lost to follow up after CR had been achieved 12 patients (42%) relapsed in the following 21 months after CR and 15 are still in the initial CR. Figure 6 shows the actuarial duration of the first CR.

It can be observed that 50% of affected patients relapsed during the first 6 months with a maximum duration of CR of 16 months, whereas in those without MI the average duration was 21 months. The curve flattens out at about 22 months when 62% of the patients had relapsed. The maximum duration was 92 months.

The *period of survival* of those patients with MI is shown in figure 7 of this group 50% died during the first 9–10 months, whereas in the group without MI the patients died during the initial 25–26 months.

No patients with MI were alive after 30 months At this point the differences with unaffected patients were statistically significant ($0.02 < p < 0.05$) The maximum period of survival of patients without MI was 93 months. It must be stressed that 4 patients who have stopped treatment belong to the group of patients not affected by MI

Table II. Cytochemical characteristics of 5 patients with ALL

Patients	Lymphoblasts in bone marrow %	Acid phosphatase, %	PAS %
Patients with mediastinal involvement			
1	90	85	10
2	96	95	0
3	94	100	0
Patients without mediastinal involvement			
4	97	0	95
5	36	4	5

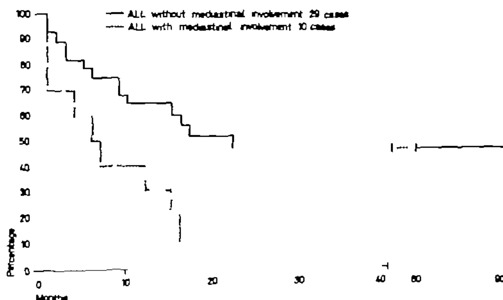


Fig 6. Actuarial display of the first complete remission and mediastinal involvement.

niques et morphologiques. Acta haemat. 2e ser. pp. 145–155 (1976).

6 Castleberry, R. P. and Toben, H. R., Moreno, H.: An evaluation of malignant cell origin in lymphosarcoma of childhood. Abstract. Blood 42: 1025 (1973).

7 Catovsky, D.; Galetto, J.; Okos, A.; Miliani, E., and Galton, D. A. G.: Cytochemical profile of B and T leukaemic lymphocytes with special reference to acute lymphoblastic leukaemia. J. clin. Path. 27: 767–771 (1974).

8 Catovsky, D.; Goldmann, J. M.; Okos, A.; Frisch, B. and Galton, D. A. G.: T-lymphoblastic leukaemia: A distinct variant of acute leukaemia. Br. med. J. ii: 643–646 (1974).

9 Catovsky, D.: T-cell origin of acid-phosphatase positive lymphoblasts. Lancet ii: 327–328 (1975).

10 Cooke, J. V.: Mediastinal tumor in acute leukemia. Am. J. Dis. Child. 44: 1153–1177 (1932).

11 Chessells, J. M.; Hardisty, R. M., and Rapson, N. T.: Acute lymphoblastic leukaemia in children. Classification and prognosis. Lancet ii: 1307–1309 (1977).

12 Esber, E.; Dinscoia, W.; Movassaghi, N. and Leikin, S.: Lymphocyte surface receptors in childhood acute lymphocytic leukemia. Pediatrics, Springfield 56: 788–792 (1975).

13 Gunz, A.; George, S. and Sullivan, M.: Unfavorable prognosis of acute leukemia in infancy. Cancer 36: 1973 (1975).

14 Greenberg, B. R.; Peter, C. R.; Glassy, F. and MacKenzie, M. R.: A case of T-cell lymphoma with convoluted lymphocytes. Cancer 38: 1602–1607 (1976).

15 Haessner, R. J.; Rosas-Uribe, A.; Wickstrum, D. A., and Smith, O. C.: Non-Hodgkin lymphoma in the first two decades of life. A pathological study of 30 cases. Cancer 40: 1533–1547 (1977).

16 Humphery, G. B. y Lankford, S.: Leucemia aguda. Clasificación con marcadores superficiales. Semin. Oncologia, Factores Pronosticos de la Leucemia Aguda, pp. 73–85 (Med. Panamericana, 1977).

17 Kaplan, J.; Mastrangelo, R., and Peterson, W. D.: Childhood lymphoblastic lymphoma. A cancer of thymus-derived lymphocytes. Cancer Res. 34: 521–525 (1974).

18 Kersey, J.; Nesbit, M.; Hallgren, H.; Sabad, A.; Yunis, E., and Gajl-Peczalska, K.: Evidence for origin of certain childhood acute lymphoblastic leukemias and lymphomas in thymus-derived lymphocytes. Cancer 36: 1348–1352 (1975).

19 Lennert, K.; Stein, H., and Kaiserling, E.: Cytological and functional criteria for the classification of malignant lymphomata. Br. J. Cancer 31: suppl. II, pp. 29–43 (1975).

20 Lukes, R. J. and Collins, R. D.: Immunologic characterization of human malignant lymphomas. Cancer 34: 1488–1503 (1974).

21 Mathé, G.; Belpomme, D. et Dantchev, D.: Hématosarcomes non Hodgkiniens. Nouv. Presse méd. 4: 241–254 (1975).

22 Miller, O. R.: Prognostic factors in childhood leukemia. J. Pediat. 87: 672 (1975).

23 Minowada, J.; Ohnuma, T. and Moore, G. E.: Rosette-forming human lymphoid cell lines. I. Establishment and evidence for origin of thymus-derived lymphocytes. J. natn. Cancer Inst. 49: 891–895 (1972).

24 Nathwani, B. N.; Kim, H., and Rappaport, H.: Malignant lymphoma, lymphoblastic. Cancer 38: 964–983 (1976).

25 Palutke, M. and Tabaczka, L.: T-cell lymphoma. Am. J. clin. Path. 64: 26–33 (1975).

26 Pantazopoulos, N. and Sinks, L. F.: Morphologic criteria for prognostication of acute lymphoblastic leukaemia. Br. J. Haemat. 27: 25 (1974).

27 Pinkel, D.; Johnson, W. and Aur, R. J. A.: Non-Hodgkin lymphoma in children. Br. J. Cancer 31: suppl. II, pp. 298–323 (1975).

28 Ravindranath, Y.; Kaplan, J. and Zuelzer, W. W.: Significance of mediastinal mass in acute lymphoblastic leukemia. Pediatrics, Springfield 55: 889–893 (1975).

29 Ritter, J.; Gaedicke, G.; Winkler, K.; Beckmann, H., and Landbeck, G.: Possible T-cell origin lymphoblasts in acid-phosphatase-positive acute lymphatic leukaemia. Lancet ii: 75 (1975).

30 Sen, L. and Borella, L.: Clinical importance of lymphoblasts with T markers in childhood acute leukemia. New Engl. J. Med. 292: 829–832 (1975).

31 Shaw, M. T.; Humphrey, G. B.; Lawrence, R., and Fischer, D. B.: Lack of prognostic value

Table III. Mediastinal involvement and cellular markers

Author	Mediastinal involvement						Patients with T markers	Patients without markers
	Patients		T		Null			
	n	%	n	%	n	%		
Catovsky et al [8]	1	14	1	33		–	3	4
Kaplan et al. [17]	3	14	3	75		–	4	8
Sen and Borella [30]	8	17	7	63	1	3	11	37
Kersey et al [18]	3	14	3	21		–	14	8
Brouet et al [5]	8	8	7	25	1	1	28	69
Tsukimoto et al [36]	5	14	5	62		–	8	28
Barrett et al [1]	8	36	4	100	4	22	4	18
Chessels et al [11]	14	15	9	82	5	6	11	81
Cooke [10]	9	24		–		–	–	–
Ravindranath et al [28]	10	18.5		–		–	–	–
Sen and Borella [30]	51	10		–		–	–	–
Pinkel et al. [27]	16	26		–		–	–	–
Nathwani et al [24]	15	50		–		–	–	–

ferences have been described by other authors [14 24 27 34 36] The mean duration of complete remission as well as the survival period of patients with mediastinal involvement in our series was clearly shorter than those without. This should stimulate us to find new and radical therapeutic methods for treating these patients.

The peculiar distribution of age, sex, MI higher aggressive behavior and worst prognosis, suggest that ALL with T markers is more closely related to mediastinal lymphomas than to ordinary ALL. At present we have not got enough data to know if the T leukemia without mediastinal involvement represents a nonthymic malignant neoplasm or if the cases of leukemia without T markers associated with mediastinal involvement correspond to a neoplasia which originates in the lymph nodes and not in the thymus.

References

1 Barrett, S. G., Schwade, J G Rankin, R, and Kadin, M. E. Lymphoblasts with both T and B markers in childhood leukemia and lymphoma. Blood *50* 71–79 (1977).

2 Bennett, J M., Catovsky D., Daniel, M. T Flandrin, G., Galton, D A. G., Gralnick, H. R., and Sultan, C. Proposals for the classification of the acute leukemias. French-American-British (FAB) co-operative group. Br J Haemat. *33* 451 (1976)

3 Borella, L. and Sen, L. T cell surface markers on lymphoblasts from acut lymphocytic leukemia. J Immun. *111* 1257–1260 (1973).

4 Borella, L. and Sen, L. E receptors on blasts from untreated acute lymphocytic leukemia (ALL). Comparison of temperature dependence of E rosettes formed by normal and leukemic lymphoid cells. J Immun. *114* 187 190 (1975).

5 Brouet, J C. Preud-Homme, J L., Flandrin, G., Valensi, F., Daniel, M T et Seligmann, M. Classification immunologique des leucémies aiguës lymphoblastiques. Corrélations cli-

Acta haemat. 62: 71-73 (1979)

Serum Glutamic Oxalaceti ansaminase, Glutamic Pyruvic Transaminase, Ga a Glutamyl Transpeptidase and Glutamic Dehydrogenase L s in Favism

Tullio Meloni, Giuseppe Pilo, Dome dusa and Angelo Dore

Children University Hospital, Sassari

Key Words. Favism · G-6-PD deficiency · Liver function in favism · GOT · GPT · Gamma-GT · GLDH

Abstract. Serum GOT, GPT, γ-GT and GLDH were determined in 15 G-6-PD-deficient subjects during a hemolytic crisis and hemoglobinuria due to ingestion of fresh fava beans. The same G-6-PD-deficient subjects were studied again 2 months after the crisis, when they were asymptomatic. 15 normal healthy children served as controls. A statistically significant increase, above normal, in serum GOT, GLDH and γ-GT was observed in the favic subjects during the crisis. All the values reverted to normal in the asymptomatic period.

The clinical picture of favism in glucose-6-phosphate dehydrogenase (G-6-PD) deficient subjects after ingestion of fresh fava beans is characterized by acute hemolytic anemia, jaundice and hemoglobinuria. A reduced ability to form prothrombin has been observed by *Vullo and Panizzon* [1] and a reduced ability to form salicylamide glucuronide has been demonstrated by *Cassimos et al.* [2] and *Cutillo et al.* [3]. Liver histology revealed cloudy and vacuole degeneration of the hepatocyte cytoplasm [1].

The present study was carried out in order to evaluate liver damage during the hemolytic crisis measuring the serum activity of glutamic oxalacetic transaminase (GOT), glutamic pyruvic transaminase (GPT), γ-glutamyl transpeptidase (γ-GT) and glutamic dehydrogenase (GLDH).

Materials and Methods

The study includes 15 G-6-PD-deficient children admitted to our hospital with severe hemolytic crisis and hemoglobinuria following the ingestion of fresh fava beans. 13 were male and 2 ere f male homozygotes. Their ages ranged from 4 to 9 years (mean 5.6). The same subjects were studied again 2 months after the hemolytic episode when they ere in good health. 15 normal children (12 males and 3 females), ages 3-10 years (mean 6), in hom hematological abnormalities had previously been excluded were ho studied and served as controls. I all cases, the diagnosis (or exclusion) of G-6-PD deficiency was made on

of the periodic acid-Schiff reaction and blast cell size in childhood acute lymphocytic leukemia. Am. J Hemat. 2 237–243 (1977).

32 Simone, J V., Verzosa, M. S and Rudy J A. Initial features and prognosis in 363 children with acute lymphocytic leukemia. Cancer *36* 2099–2108 (1975)

33 Smith, J L. Barker C. R., Cleine, G., P and Collins, R. D Characterisation of malignant mediastinal lymphoid neoplasm (Sternberg Sarcoma) as thymic in origin. Lancet *I.* 74–77 (1973)

34 Stathopoulus, G., Davies, A. J S. Papamichail, M., Holborow E. J and Wiltshaw E. Origin of malignant cells in lymphoma a apparently originated in anterior mediastinum. Lancet *I.* 74–77 (1973)

35 Stein, H Petersen, N., Gaedicke, G., Lennert, K., and Landbeck, G Lymphoblastic lymphoma of convoluted or acid phosphatase type A tumor of T precursor cells. Int. J Cancer *17* 92–295 (1976)

36 Tsukimoto, I Wong, K. Y and Lampkin, B. C. Surface markers and prognostic factors in acute lymphoblastic leukemia. New Engl. J Med *294* 245–248 (1976)

Received. August 25 1978
Accepted December 29 1978

Dr E. Conde, Department of Haematology of the Marqués de Valdecilla Medical Centre, Santander (Spain)

In our opinion, the high γ-GT and GLDH levels during the hemolytic crisis could be due to intrahepatic cholestasis in spite of the low proportions of conjugated bilirubin. The high levels of GOT however besides cholestasis, could be related to hemolysis. In the asphyxia increased serum GOT is associated to an increase of GPT too, as was recently shown in animal experiments [8].

This is also supported by the fact that the elevation in serum enzyme is short lived ad completely reversible, since 2 months after the hemolytic crisis all values had returned to normal.

References

1 Vullo, C. Panizon, F. Aspetti istologici funzionali del fegato nel favismo emolitico. Acta paediat. lat. 8. 381 389 (1955)

2 Cassimos, C., Malaka-Zafiriu, K., Tsiures, L. and Danielides, B. Variations in salicylamide glucuronide formation in normal and in G-6-PD-deficient children. J Pediat. 84 110–111 (1974).

3 Cudillo, S., Costa, S., Vinculeddu, M. C., and Meloni, T. Salicylamide-glucuronide formation in children with favism and in their parents. Acta haemat. 44 296–299 (1976).

4 Sansone, G. Rasore-Quartino, A. e Veneziano, G. Dimostrazione su strisci di sangue di una doppia popolazione eritrocitaria nelle donne eterozigoti per la deficienza in glucosio-6-P deidrogenasi. Pathologica 55 371 375 (1963).

5 Malloy H. T. and Evelyn, K. A. Determination of bilirubin with the photoelectric colorimeter J biol. Chem. 119 481–490 (1937).

6 Kattamis, C., Karambula, K., Ioannidou, V and Hatzikou, V. Jaundice and bilirubin levels in Greek children with favism. Archs. Dis. Childh. 51 233-235 (1976).

7 Peracino, A. Marcovina, S. Valutazione critica di alcune correlazioni morfologiche sieroenzimatiche di frazioni plasmoproteiche nelle affezioni epatiche. Quad. Sclavo Diagn. 8 310–337 (1972).

8 Sawaguchi, A. and Yoshimori, K. A study on rabbit serum transaminase activity in asphyxia. Jap. J leg. Med. 23 409–414 (1969).

Received October 4 1978
Accepted: February 14, 1979

Dr Tullio Meloni, Clinica Pediatrica dell'Università, Viale San Pietro 12, I-07100 Sassari (Italy)

venous blood using the cytochemical test for *Sansone et al.* [4 this being done in subjects with hemolytic crisis immediately before any blood transfusion]

Blood hemoglobin concentration was determined by Drabkin's method blood hematocrit was measured using Ljumberg Cellocrite microhematocrit tubes centrifuged for 10 min at 10 000 rpm serum bilirubin determinations were made by *Malloy and Evelyn's* [5] method. Serum GOT GPT γ-GT and GLDH were determined using Boehringer monotests.

Results

The results obtained in the three groups of children studied are shown in table I

As shown in the table, the mean values of serum GOT γ-GT GLDH, total and conjugated bilirubin were significantly ($p < 0\ 001$) higher in G-6-PD-deficient subjects during the hemolytic crisis when compared to the values obtained in the same subjects during the asymptomatic period or to those obtained in normal controls. The values in the two latter groups of subjects did not differ significantly The mean serum GPT values were similar in all three groups of subjects studied.

Discussion

Kattamis et al [6] found increased serum GOT and GPT values in G-6-PD deficient children during the hemolytic crisis and attributed this finding to erythrocyte lysis.

In our cases, we found increased levels of serum GOT but normal levels of serum GPT Therefore, the increased levels of serum GOT cannot be attributed only to hemolysis since there was no corresponding increase in serum GPT Furthermore, in addition to the increase in serum GOT we observed an increase in γ-GT and GLDH, which is clearly of hepatic origin. High serum GOT γ-GT and GLDH values and normal serum GPT values were also observed by *Peracino and Marcovina* [7] in subjects with liver changes characterized by cloudy and vacuole degeneration of liver cells and showing evidence of cholestasis. Similar histological findings without evidence of cholestasis have been reported by *Vullo and Panizon* [1] in G-6-PD-deficient subjects during the hemolytic phase. They ascribe these changes to the asphyxia secondary to anemia.

Table I. Mean values (± SD) of serum GOT GPT γ-GT GLDH total and conjugated bilirubin in G-6-PD-deficient subjects during a hemolytic crisis (group 1) and 2 months later (group 2) The values obtained in 15 normal children are also shown for comparison (group 3)

Subjects	SGOT U/l	SGPT U/l	γ-GT U/l	GLDH, U/l	Total bilirubin mg/dl	Conjugated bilirubin mg/dl
Group 1	53 46 ± 32.61	9.86 ± 4 06	20 06 ± 3.97*	27 60 ± 18 18	5 17 ± 2.81	1.00 ± 0.41
Group 2	12.66 ± 3 63	9.80 ± 2.93	10.53 ± 4 68	4.86 ± 1.84	0.80 ± 0.17	0.24 ± 0.15
Group 3	13.93 ± 2.91	9 13 ± 3.81	11 73 ± 4 78	5 40 ± 1 68	0.74 ± 0.15	0.26 ± 0.13

Values significantly higher than in the other two groups ($p < 0.001$).

In our opinion, the high γ-GT and GLDH levels during the hemolytic crisis could be due to intrahepatic cholestasis, in spite of the low proportions of conjugated bilirubin. The high levels of GOT however besides cholestasis, could be related to hemolysis. In the asphyxia increased serum GOT is associated to an increase of GPT too, as was recently shown in animal experiments [8]

This is also supported by the fact that the elevation in serum enzyme is short lived ad completely reversible, since 2 months after the hemolytic crisis all values had returned to normal.

References

1 Vallo, C. Panizon, F. Aspetti istologici funzionali del fegato nel favismo emolitico. Acta paediat. lat. 8, 381–389 (1955).
2 Cassimos, C., Malaka-Zafiriu, K., Tsiures, J. and Danielides, B. Variations in salicylamide glucuronide formation in normal and in G-6-PD-deficient children. J. Pediat. 84 110–111 (1974).
3 Cutillo, S., Costa, S., Vintuleddu, M. C., and Meloni, T. Salicylamide-glucuronide formation in children with favism and in their parents. Acta haemat. 44 296–299 (1976).
4 Samsone, G. Rasore-Quartino, A. Veneziano, G. Dimostrazione su strisci di sangue di una doppia popolazione eritrocitaria nelle donne eterozigoti per la deficienza in glucosio-6-P deidrogenasi. Pathologica 55 371 375 (1963).
5 Malloy H. T. and Evelyn, K. A. Determination of bilirubin with the photoelectric colorimeter. J. biol. Chem. 119 481–490 (1937).
6 Kattamis, C. Karambula, K., Ioannidou, V. and Hatzikou, V. Jaundice and bilirubin levels in Greek children with favism. Archs. Dis. Childh. 51 233–235 (1976).
7 Perasino, A. Marcovina, S. Valutazione critica di alcune correlazioni morfologiche siero-enzimatiche di frazioni plasmoproteiche nelle affezioni epatiche. Quad. Sclavo Diagn. 8 310–337 (1972)
8 Sawaguchi, A. and Yoshimari, K. A study on rabbit serum transaminase activity in asphyxia. Jap. J. leg. Med. 23 409–414 (1969).

Received October 4, 1978
Accepted: February 14, 1979

Dr. Tullio Meloni, Clinica Pediatrica dell'Università, Viale San Pietro 12, I-07100 Sassari (Italy)

venous blood using the cytochemical test for *Sansone et al* [4 this being done in subjects with hemolytic crisis immediately before any blood transfusion]

Blood hemoglobin concentration was determined by Drabkin's method blood hematocrit was measured using Ljumberg Cellocrite microhematocrit tubes centrifuged for 10 min at 10 000 rpm serum bilirubin determinations were made by *Malloy and Evelyn's* [5] method. Serum GOT GPT γ-GT and GLDH were determined using Boehringer monotests.

Results

The results obtained in the three groups of children studied are shown in table I

As shown in the table, the mean values of serum GOT γ-GT GLDH, total and *conjugated bilirubin were significantly ($p < 0.001$) higher in G-6-PD-deficient subjects* during the hemolytic crisis when compared to the values obtained in the same subjects during the asymptomatic period or to those obtained in normal controls. The values in the two latter groups of subjects did not differ significantly The mean serum GPT values were similar in all three groups of subjects studied.

Discussion

Kattamis et al [6] found increased serum GOT and GPT values in G-6-PD deficient children during the hemolytic cr... and attributed this finding to er... lysis.

In our cases, we found increased ... of serum GOT but normal levels of se... GPT Therefore, the increased levels of serum GOT cannot be attributed only to he... lysis since there was no corresponding increase in serum GPT Furthermore, in addition to the increase in serum GOT we observed an increase in γ-GT and GLDH, which is clearly of hepatic origin High serum GOT γ-GT and GLDH values and normal serum GPT values were also observed by *Peracino and Marcovina* [7] in subjects with liver changes characterized by cloudy and vacuole degeneration of liver cells and showing evidence of cholestasis. Similar histological findings without evidence of cholestasis have been reported by *Vullo and Panizon* [1] in G-6-PD-deficient subjects during the hemolytic phase They ascribe these changes to the asphyxia secondary to anemia.

Table I. Mean values (± SD) of serum GOT GPT γ-GT GLDH total and conjugated bilirubin in G-6-PD-deficient subjects during a hemolytic crisis (group 1) and 2 months later (group 2) The values obtained in 15 normal children are also shown for comparison (group 3)

Subjects	SGOT U/l	SGPT U/l	γ-GT U/l	GLDH, U/l	Total bilirubin mg/dl	Conjugated bilirubin mg/dl
Group 1	53 46 ± 32.61	9 86 ± 4 06	20.06 ± 3.97*	27 60 ± 18.18	5 17 ± 2.81	1 00 ± 0.41
Group 2	12 66 ± 3 63	9.80 ± ..93	10.53 ± 4 68	4.86 ± 1 84	0.80 ± 0.17	0.24 ± 0.15
Group 3	13.93 ± 2.91	9 13 ± 3.81	11 73 ± 4 78	5 40 ± 1.68	0.74 ± 0 15	0.26 ± 0.13

* Values significantly higher than in the other two groups ($p < 0.001$).

In our opinion, the high γ-GT and GLDH levels during the hemolytic crisis could be due to intrahepatic cholestasis, in spite of the low proportions of conjugated bilirubin. The high levels of GOT however besides cholestasis, could be related to hemolysis. In the asphyxia increased serum GOT is associated to an increase of GPT too, as was recently shown in animal experiments [8]

This is also supported by the fact that the elevation in serum enzyme is short lived ad completely reversible, since 2 months after the hemolytic crisis all values had returned to normal.

References

1 Vullo, C. Panizon, F Aspetti istologici funzionali del fegato nel favismo emolitico Acta paediat. lat 8. 381 389 (1955)

2 Cassimos, C., Malaka-Zafiriu, K., Tsiures, L. and Danielides, B. Variations in salicylamide glucuronide formation in normal and in G-6-PD-deficient children. J Pediat. 84 110–111 (1974).

3 Cotillo, S., Costa, S Vinteleddu, M. C., and Meloni, T Salicylamide-glucuronide formation in children with favism and in their parents. Acta haemat. 44 296–299 (1976).

4 Sansone, G Rasore-Quartino, A. Veneziano, G. Dimostrazione su strisci di sangue di una doppia popolazione eritrocitaria nelle donne eterozigoti per la deficienza in glucosio-6-P deidrogenasi. Pathologica 55 371–375 (1963).

5 Malloy H. T and Evelyn, K. A. Determination of bilirubin with the photoelectric colorimeter J biol. Chem. 119 481–490 (1937).

6 Kattamis, C., Karambula, K., Ioannidou, V and Hatzikou, V Jaundice and bilirubin levels in Greek children with favism Archs. Dis. Childh. 51 233–235 (1976).

7 Peracino, A. Marcovina, S. Valutazione critica di alcune correlazioni morfologiche siero-enzimatiche di frazioni plasmoproteiche nelle affezioni epatiche. Quad. Sclavo Diagn. 8. 310–337 (1972).

8 Sawaguchi, A. and Yoshinari, K. A study on rabbit serum transaminase activity in asphyxia. Jap. J leg. Med. 23 409–414 (1969).

Received October 4, 1978
Accepted February 14, 1979

Dr Tullio Meloni, Clinica Pediatrica dell'Università, Viale San Pietro 12, I-07100 Sassari (Italy)

venous blood using the cytochemical test for *Sansone et al* [4 this being done in subjects with hemolytic crisis immediately before any blood transfusion].

Blood hemoglobin concentration was determined by Drabkin's method blood hematocrit was measured using Ljumberg Cellocrite microhematocrit tubes centrifuged for 10 min at 10,000 rpm serum bilirubin determinations were made by *Malloy and Evelyn's* [5] method. Serum GOT GPT γ-GT and GLDH were determined using Boehringer monotests.

Results

The results obtained in the three groups of children studied are shown in table I

As shown in the table, the mean values of serum GOT γ-GT GLDH, total and conjugated bilirubin were significantly ($p < 0.001$) higher in G-6-PD-deficient subjects during the hemolytic crisis when compared to the values obtained in the same subjects during the asymptomatic period or to those obtained in normal controls. The values in the two latter groups of subjects did not differ significantly The mean serum GPT values were similar in all three groups of subjects studied.

Discussion

Kattamis et al [6] found increased serum GOT and GPT values in G-6-PD-deficient children during the hemolytic crisis and attributed this finding to erythrocyte lysis.

In our cases, we found increased levels of serum GOT but normal levels of serum GPT Therefore the increased levels of serum GOT cannot be attributed only to hemolysis since there was no corresponding increase in serum GPT Furthermore, in addition to the increase in serum GOT we observed an increase in γ-GT and GLDH, which is clearly of hepatic origin. High serum GOT γ-GT and GLDH values and normal serum GPT values were also observed by *Peracino and Marcovina* [7] in subjects with liver changes characterized by cloudy and vacuole degeneration of liver cells and showing evidence of cholestasis. Similar histological findings without evidence of cholestasis have been reported by *Vullo and Panizon* [1] in G-6-PD-deficient subjects during the hemolytic phase. They ascribe these changes to the asphyxia secondary to anemia.

Table I. Mean values (± SD) of serum GOT GPT γ-GT GLDH total and conjugated bilirubin in G-6-PD-deficient subjects during a hemolytic crisis (group 1) and 2 months later (group 2). The values obtained in 15 normal children are also shown for comparison (group 3)

Subjects	SGOT U/l	SGPT U/l	γ-GT U/l	GLDH U/l	Total bilirubin mg/dl	Conjugated bilirubin mg/dl
Group 1	53 46 ± 32.61	9.86 ± 4.06	20.06 ± 3 97	27 60 ± 18 18	5.17 ± -.81	1.00 ± 0.41
Group 2	12.66 ± 3 63	9 80 ± 2.93	10.53 ± 4 68	4 86 ± 1.84	0.80 ± 0.17	0.24 ± 0.15
Group 3	13.93 ± 2.91	9 13 ± 3.81	11 73 ± 4 78	5.40 ± 1 68	0.74 ± 0 15	0.26 ± 0.13

Values significantly higher than in the other two groups ($p < 0.001$).

blood by centrifugation in an International centrifuge at 85 and 850 g respectively for 10 min. For platelet counts, blood was collected with EDTA heparin. Platelet counts [5] were carried out using Zeiss microscope and an Improved Neubauer chamber. The normal range was 150,000–400 000 /µl.

Platelet aggregation was induced by ADP ad enosine 5-diphosphate disodium salt, Sigma Chemical Co.) and by arachidonic acid (acid arachidon ic sodium salt No. 1391, Sigma Chemical C). The two substances were dissolved in sodium barbital buffer pH 7.35 t give final concentration of 6.2×10^{-4} and 3 mM respectively.

Platelet aggregation induced by ADP and arachidonic acid was examined with an Evans lectroelenium aggregation meter (EEL), with built in stirrer model 169 by the method of *Born and Cross* [1]. Platelet-rich plasma was adjusted to $200{,}000 \pm 20{,}000$/ l, and 0.6 ml, warmed at 37 °C, were aggregated for 6 min. Determinations in both groups, patients and controls, were carried out without and with addition of 50 µl D, L-α-tocopherol of 500 mg/ml concentration in ethanol 95 incubated 10 min at 37 °C before aggregation, or only with addition of 50 l ethanol [16]. The platelet-poor plasma was considered as 100% and the platelet-rich plasma as 0% of aggregation. The maximal aggregation after 4 min was calculated and the results were given in percent. The normal maximal ADP-induced aggregation ranged from 70 to 100%. There was no effect of the arachidonic acid on platelet aggregation in the control subjects.

The clot retraction inhibition test [6] was carried out with the same aggregating substances as described before and with the same concentrations. Platelet-rich plasma without and with addition f 50 µl D, L-α-tocopherol of 500 mg/dl was used, as well as 50 l ethanol only and the results of the degree of clot retraction inhibition reported after half an hour incubation at 37 C. The normal range was 50–75% with ADP and 80–105% with arachidonic acid.

Results

The results obtained in the normal and diabetic patients are presented in tables I and II. The platelet count in the control

Table I. Platelet aggregation induced by ADP and arachidonic acid before and after incubation with vitamin E

	Platelet count/µl	Platelet aggregation, %			
		ADP	ADP + vitamin E	arachidonic acid	arachidonic acid + vitamin E
Normal subjects	207,000 ± 12,500	89.6 ± 4.92	43.5 ± 6.82	no aggregation	45.3 ± 5.28
Diabetic retinopathy patients	160,000 ± 15,200	85.0 ± 4.20	42.3 ± 5.40	14.9 ± 6.10	41.2 ± 6.70

Table II. Clot retraction inhibition test with ADP and arachidonic acid before and after incubation with vitamin E

	Degree of inhibition of clot retraction, %			
	ADP	ADP + vitamin E	arachidonic acid	arachidonic acid + vitamin E
Normal subjects	70 ± 3.00	19.0 ± 5.20	100 ± 2.40	87.5 ± 2.00
Diabetic retinopathy patients	65 ± 2.00	15.0 ± 3.60	96 ± 1.50	88.0 ± 3.20

Acta haemat. 62 74–77 (1979)

Effect of Vitamin E on Platelet Aggregation in Diabetic Retinopathy

Draga Creter Fira Pavlotsky and Hanna Savir[1]

Departments of Hemostasis and Ophthalmology Hasharon Hospital, Petah-Tiqva

Key Words. Adenosine diphosphate Arachidonic acid Diabetic retinopathy Platelet aggregation Vitamin E

Abstract. The effect of vitamin E on platelet aggregation was investigated in a group of 10 patients with diabetic retinopathy Adenosine diphosphate induced platelet aggregation was inhibited in the patients group as well as in the controls in the presence of vitamin E. An increased platelet aggregation was obtained with arachidonic acid in both groups when platelet rich plasma was incubated prior with vitamin E. The clot retraction inhibition test parallels the findings of aggregation obtained with the platelet aggregation meter

Vitamin E (tocopherol) comprises a few liposoluble factors with multiple activities [3 17] The main tocopherols are α, β γ and δ related to the change of the radicals of the group and α- β γ- and δ-tocotrienols α-Tocopherol possesses the greatest biological activity [17] The tocopherols are potent natural intracellular antioxidants and have also a vasodilatator action [3] The increase of lipid peroxides normally associated with platelet aggregation is markedly reduced by α-tocopherol similarly to acetyl salicylic acid, a known inhibitor of the platelet release reaction [16]

The authors wish to thank Prof *M Djaldetti* for his valuable advice and help throughout the preparation of the manuscript.

The purpose of the present study was to examine the effect of vitamin E on platelet aggregation in a group of patients with diabetic retinopathy A good correlation between platelet aggregation activity and diabetic retinopathy was found and a possible role in the genesis of diabetic microangiopathy is suggested [12] Inhibition of platelet aggregation by vitamin E may serve as a possible tool in the prevention and treatment of thrombotic events.

Materials and Methods

10 patient with diabetic retinopathy and 10 healthy donors were studied. Platelet rich and poor plasma was obtained from citrated venous

blood by centrifugation in an International centrifuge at 85 and 850 g respectively for 10 min. For platelet counts, blood was collected with EDTA heparin. Platelet counts [5] were carried out using Zeiss microscope and an improved Neubauer chamber. The normal range was 150,000–400,000 µl.

Platelet aggregation was induced by ADP adenosine-5'-diphosphate disodium salt, Sigma Chemical Co.) and by arachidonic acid (acid arachidonic sodium salt No. 1391 Sigma Chemical C) The two substances were dissolved in sodium barbital buffer pH 7.35, to give final concentration of 6.2×10^{-4} and 3 mM respectively.

Platelet aggregation induced by ADP and arachidonic acid was examined with an Evans electroselenium aggregation meter (EEL), with bushlo stirrer, model 169 by the method of *Born and Cross* [1]. Platelet-rich plasma was adjusted to 200,000 ± 20,000 µl, and 0.6 ml, warmed t 37 C, were aggregated for 6 min. Determinations in both groups, patients and controls, were carried out without and with addition of 50 l *D L*-α-tocopherol of 500 mg/ml concentration in ethanol 95% incubated 10 min at 37 C before aggregation, or only with addition of 50 l ethanol [16]. The platelet-poor plasma was considered as 100% and the platelet-rich plasma as 0% of aggregation. The maximal aggregation after 4 min was calculated and the results were given in percent. The normal maximal ADP-induced aggregation ranged from 70 to 100%. There was no effect of the arachidonic acid on platelet aggregation in the control subjects.

The clot retraction inhibition test [6] was carried out with the same aggregating substances as described before and with the same concentrations. Platelet-rich plasma without and with addition of 50 µl *D L*-α-tocopherol of 500 mg/dl was used, as well as 50 l ethanol only and the results of the degree of clot retraction inhibition reported fter half an hour incubation at 37 °C. The normal range was 50–75% with ADP and 80–105% with arachidonic acid.

Results

The results obtained in the normal and diabetic patients are presented in tables I and II The platelet count in the control

Table I. Platelet aggregation induced by ADP and arachidonic acid before and after incubation with vitamin E

	Platelet count/µl	Platelet aggregation, %			
		ADP	ADP + vitamin E	arachidonic acid	arachidonic acid + vitamin E
Normal subjects	207,000 ± 12,500	89.6 ± 4.92	43.5 ± 6.82	no aggregation	45.3 ± 5.28
Diabetic retinopathy patients	160,000 ± 15,200	85.0 ± 4.20	42.3 ± 5.40	14.9 ± 6.10	41.2 ± 6.70

Table II. Clot retraction inhibition test with ADP and arachidonic acid before and after incubation with vitamin E

	Degree of inhibition of clot retraction, %			
	ADP	ADP + vitamin E	arachidonic acid	arachidonic acid + vitamin E
Normal subjects	70 ± 3.00	190 ± 5.20	100 ± .40	87.5 ± 00
Diabetic retinopathy patients	68 ± 2.00	190 ± 3.60	96 ± 1.50	58.0 ± 3.20

subjects was within the normal range The diabetic patients showed a slight deviation from the normal, being below or at the lower limit of the normal range (table I)

ADP induced platelet aggregation was inhibited in the presence of vitamin E in patients and controls No aggregation was obtained with arachidonic acid in the control group, whereas an increased platelet aggregation was obtained when platelet rich plasma was incubated prior with vitamin E, in patients and controls (table I) No special inhibitory effect was found when ethanol was used in the specificated quantities. ADP and α-tocopherol induced a more pronounced inhibition of clot retraction When arachidonic acid was used alone or with α-tocopherol the results of the degree of inhibition were quite the same in both groups (table II)

Discussion

Platelets are unique for their ability to synthesize protein and glycogen, as well as lipids and fatty acids [15] These cells are capable of a *de novo* phospholipid and fatty acid synthesis [15] It has been shown that platelet aggregation increases lipid and amino acid synthesis [15] The process of aggregation and inhibition of the release reaction in the presence of vitamin E – a strong antioxidant – is related to the membrane and subcellular organelles [16] According to the antioxidant hypothesis [13] the only function of vitamin E in biological systems is an antioxidant and the molecules protected from oxidation are unsaturated lipids. On the other hand, vitamin E fulfills functions other than that of an antioxidant, namely it has a physicochemical role in the stabilization of those biological membranes that contain high levels of polyunsaturated fatty acids [13] The physical properties of the molecule merit no less consideration than its chemical behavior as an antioxidant [13] According to the molecular models, vitamin E stabilizes membranes by virtue of specific physicochemical interactions between its phytyl side chain and the fatty acyl chains of polyunsaturated phospholipids, particularly those derived from arachidonic acid [13] Vitamin E may interact with the arachidonic acid residues located in the membrane, either in relation to phospholipids, or in relation to membrane proteins [13] It is suggested to consider the role of vitamin E in terms of its mediation of specific hydrogen transfer or electron transfer reactions at certain membrane sites [9]

The inhibitors of platelet aggregation are widely used in the prevention of thrombotic events. Microangiopathy and vasoocclusive disease are recognized complications of diabetes mellitus and platelets have probably a role in the pathogenesis of these disorders [14] Platelets from patients with diabetes showed increased sensitivity to low concentrations of ADP [2, 12] This confirms other studies but, however the lack of correlation with complications and the wide range of sensitivities found in normal subjects makes the interpretation of the results difficult [2]

The platelet aggregation in diabetic retinopathy was previously described. No statistically significant increased sensitivity to ADP was found by us in the normal group as compared with the diabetic retinopathy patients [4] The present study demonstrates that ADP induced aggregation in the presence of vitamin E was inhibited This effect was not obtained when ADP was substituted by arachidonic acid.

Khurshid et al. [11] have shown an ab- platelet ristocetin aggregation in a 16-month-old child with vitamin E deficiency chronic jaundice and hepatic fibrosis. The defective platelet function was corrected by treatment with vitamin E. *Gomes et al.* [8] have not found significant changes in platelet aggregation when using α-tocopherol. They suggested that the beneficial effect of vitamin E in the prevention and treatment of thromboembolic phenomena is not the result of the tocopherol effect on platelet aggregation.

The question whether the present results are due to the antioxidant effect of vitamin E on the release of platelet aggregation, or to an association with the membrane structure acting by another mechanism [16] remains unanswered. Further studies on the metabolism of prostaglandin – endoperoxide [7, 10] derived from the arachidonic acid will help to elucidate the role of vitamin E.

References

1 Born, G. V. R. and Cross, M. J.: The aggregation of blood platelets. J. Physiol., Lond. *168*: 178–195 (1963).

2 Burrows, A. W.; Chavin, S. L., and Hockaday, T. D. R.: Plasma thromboglobulin concentrations in diabetes mellitus. Lancet: 235–237 (1978).

3 Courtois, J. E. et Perles, R.: Précis de chimie biologique (Masson, Paris 1964).

4 Creter, D.; Pavlotzky, F. and Savir, H.: Platelet aggregation in diabetic retinopathy. Acta haemat. *60*: 53–55 (1978).

5 Dacie, J. V. and Lewis, S. M.: Practical haematology; 5th ed. (Churchill Livingstone, Edinburgh 1975).

6 De Gaetano, G.; Vermylen, J. and Verstraete, M.: A simple indirect method to evaluate platelet aggregation. The clot retraction inhibition (CRI) test. Thromb. Diath. haemorrh. *29*: 660–670 (1973).

7 Editorial. Prevention of thrombosis. Lancet *i*: 127–128 (1977).

8 Gomes, J. A. C.; Venkatachalapathy, D. and Haft, J. L.: The effect of vitamin E on platelet aggregation. Am. Heart J. *91*: 425–429 (1976).

9 Green, J.: Vitamin E and the biological antioxidant theory. Ann. N.Y. Acad. Sci. *203*: 29–44 (1972).

10 *Holmsen, H.*: Prostaglandin endoperoxide – thromboxane synthesis and dense granule secretion as positive feedback loops in the propagation of platelet responses during 'The basic platelet reaction'. Thromb. Haemostasis *38*: 1030–1041 (1977).

11 Khurshid, M.; Lee, T. J.; Peake, I. R., and Bloom, A. L.: Vitamin E deficiency and platelet functional defect in a jaundiced infant. Br. med. J. *iv*: 19–21 (1975).

12 Kwaan, H. C.; Colwell, J. A.; Cruz, S.; Suwanwela, N. and Dobbie, J. G.: Increased platelet aggregation in diabetes mellitus. J. Lab. clin. Med. *80*: 236–246 (1972).

13 Lucy, J. A.: Functional and structural aspects of biological membranes: suggested structural role for vitamin E in the control of membrane permeability and stability. Ann. N.Y. Acad. Sci. *203*: 4–11 (1972).

14 Preston, F. E.; Ward, J. D.; Marcola, B. H.; Porter, N. R., and Timperley, W. R.: Elevated β-thromboglobulin levels and circulating platelet aggregates in diabetic microangiopathy. Lancet: 238–239 (1978).

15 Steiner, M.: Lipid and amino acid synthesis in aggregating platelets. Ann. N.Y. Acad. Sci. *201*: 92–101 (1972).

16 Steiner, M. and Anastasi, J.: Vitamin E. An inhibitor of the platelet release reaction. J. clin. Invest. *57*: 732–737 (1976).

17 Wasserman, R. H. and Taylor, A. N.: Metabolic roles of fat-soluble vitamins D, E, and K. A. Rev. Biochem. *41*: 179–202 (1972).

Received: December 27, 1978
Accepted: March 2, 1979

Draga Creter, MSc,
Department of Hemostasis, Hasharon Hospital,
Petah-Tiqva (Israel)

Acta haemat. 62 78–80 (1979)

Multiple Myeloma and Acute Leukemia

George A Kastanas Charilaos A Papaioannou and Helen A Nathena

2nd Internal Medicine Department, Internal Medical Department and Department of Haematology Army Share Fund Hospital, Athens

Key Words. Acute leukemia Multiple myeloma

Abstract. A patient suffering from multiple myeloma without treatment with cytotoxic drugs or irradiation, died within 4 months from acute leukemia The appearance of acute leukemia in a patient with multiple myeloma without previous treatment is very unusual. The relation of these two conditions is briefly discussed.

The development of acute leukemia (AL) in patients suffering from multiple myeloma (MM) has been reported in over 60 cases [1–21] Most of these patients had received cytotoxic drugs such as cyclophosphamide and/or melfalan irradiation or a combination of both. The development of AL in these cases has been attributed to the therapy

The association of MM and AL, in patients without any previous treatment has been reported in only 3 cases [5 20]

We report here a patient with MM for which no treatment was given and who developed AL 4 months after the initial diagnosis.

Case Report

A male aged 70 years was admitted to a Community Hospital for progressive wasting during the last month prior to admission. The blood examination revealed severe anemia hematocrit = 12% hemoglobin = 3.8 g/dl WBC = 6,100/ l with 53% neutrophils, 39% lymphocytes, 3% stab cells, 4 % large mononuclears, 1 % eosinophils and 216,000/ l platelets. The patient received two units of blood and was thereafter referred to our unit.

On admission, the clinical examination apart from intense pallor revealed no abnormality Laboratory examinations hematocrit = 20%, hemoglobin = 3.6 g/dl WBC = 3,800/ l with 32% neutrophils, 56% lymphocytes, 8% mononuclears 4 % eosinophils, 150,000/μl platelets and 0.5% reticulocytes. The serum protei electrophoresis showed a narrow spike (fig. 1), which was identified on immunoelectrophoresi as a monoclonal IgG Th remaining routine laboratory tests revealed no bnormality The myelogram showed 30% plasma cells of which 8% were abnormal Bence-Jones protein was not found n the urine and the X-ray survey of the bones was negative

In view of the above the diagnosis of MM was established.

The patient received blood transfusions and 40 mg of prednisone daily On the 11th day of hospitalization he developed left-sided lung conso-

lidation for which treatment with Gentamicin was instituted.

The patient left the hospital of his own will. On discharge his blood count was as follows: hematocrit = 35%, WBC = 1,800/µl with 22% neutrophils, 73% lymphocytes, 2% eosinophils, 3% large monocytes and 150,000 µl platelets.

The patient returned 4 months later complaining of extreme weakness and high fever (38.5 °C). Outside the hospital the patient received no treatment. On examination, apart from extreme pallor no other abnormality was revealed. The blood count was as follows: hematocrit = 28%, WBC = 10,000/µl, 15% blasts, 10% neutrophils, 30% lymphocytes, 40% large monocytes, 5% metamyelocytes and 60,000/ l platelets. The myelogram showed marked infiltration of the bone marrow by immature blasts, while the electrophoresis and immunoelectrophoresis did not differ from that of his previous admission. The diagnosis of AL was established. As cytochemical studies were, unfortunately, not performed the exact type of AL cannot be identified with certainty. The patient died 15 days following his second admission. The peripheral blood picture 24 h before death was: hematocrit = 25%; WBC = 190,000 µl of which 90% blasts, 2% neutrophils, 4% lymphocytes, 4% large mononuclears and 150,000/ l platelets.

Discussion

AL has been observed in a number of cases as a final event in patients suffering from MM.

The first well-documented case was reported in 1966 by *Osserman and Lawlor* [1] in a patient aged 44 who developed monomyelocytic leukemia, 48 months after following the diagnosis of MM for which he had received treatment with melfalan, urethane and irradiation. A good number of similar reports has followed [–21]. This has led to the hypothesis that treatment may be responsible for the development of AL in patients suffering from MM [3].

The first case of MM

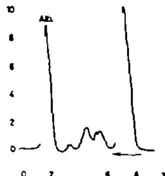


Fig. 1. Serum electrophoretic pattern showing monoclonal spike in the gamma region.

AL without any treatment was described by *Videback* [5] in 1971. *Tursz et al.* [20] reported another two similar cases of MM followed by AL in which no treatment for the myeloma was given. The present case is similar to those described by *Videback and Tursz*. We believe that in our case the infiltration of the bone marrow by numerous plasma cells, some of which were immature, together with monoclonal gammopathy leaves no doubt about the diagnosis of myeloma. It is regrettable that owing to lack of facilities at that time the study of the case was incomplete. In the cases described in the literature the AL was of the myelomonocytic type. In the present case the type of AL was not identified, as neither histochemical techniques were performed nor lysozyme was determined in the serum and the urine.

References

1 Osserman, E. F. and Lawlor, D. P.: Serum and urinary lysozyme (muramidase) in monocytic and monomyelocytic leukemia. J. exp. Med.

Acta haemat. 62: 78–80 (1979)

Multiple Myeloma and Acute Leukemia

George A. Kastanas, Charilaos A. Papaioannou and Helen A. Nathena

2nd Internal Medicine Department, Internal Medical Department and Department of Haematology, Army Share Fund Hospital, Athens

Key Words. *Acute leukemia · Multiple myeloma*

Abstract. A patient suffering from multiple myeloma without treatment with cytotoxic drugs or irradiation, died within 4 months from acute leukemia. The appearance of acute leukemia in a patient with multiple myeloma without previous treatment is very unusual. The relation of these two conditions is briefly discussed.

The development of acute leukemia (AL) in patients suffering from multiple myeloma (MM) has been reported in over 60 cases [1–21]. Most of these patients had received cytotoxic drugs such as cyclophosphamide and/or melfalan, irradiation or a combination of both. The development of AL in these cases has been attributed to the therapy.

The association of MM and AL, in patients without any previous treatment, has been reported in only 3 cases [5, 20].

We report here a patient with MM for which no treatment was given and who developed AL 4 months after the initial diagnosis.

Case Report

A male aged 70 years was admitted to a Community Hospital for progressive wasting during the last month prior to admission. The blood examination revealed severe anemia: hematocrit = 12%, hemoglobin = 3.8 g/dl, WBC = 6,100/μl with 53% neutrophils, 39% lymphocytes, 3% stab cells, 4% large mononuclears, 1% eosinophils and 216,000/μl platelets. The patient received two units of blood and was thereafter referred to our unit.

On admission, the clinical examination apart from intense pallor revealed no abnormality. Laboratory examinations: hematocrit = 20%, hemoglobin = 3.6 g/dl, WBC = 3,800/μl with 32% neutrophils, 56% lymphocytes, 8% mononuclears, 4% eosinophils, 150,000/μl platelets and 0.5% reticulocytes. The serum protein electrophoresis showed a narrow spike (fig. 1), which was identified on immunoelectrophoresis as a monoclonal IgG. The remaining routine laboratory tests revealed no abnormality. The myelogram showed 30% plasma cells of which 8% were abnormal. Bence-Jones protein was not found in the urine and the X-ray survey of the bones was negative.

In view of the above the diagnosis of MM was established.

The patient received blood transfusion and 40 mg of prednisone daily. On the 11th day of hospitalization he developed left-sided lung conso-

lidation for which treatment with Gentamicin was instituted.

The patient left the hospital of his own will. On discharge his blood count was as follows: hematocrit = 35% WBC = 1,800/μl with 22% neutrophils, 73% lymphocytes, 2% eosinophils, 3% large monocytes and 150,000/μl platelets.

The patient returned 4 months later complaining of extreme weakness and high fever (38.5 °C). Outside the hospital the patient received no treatment. On examination, apart from extreme pallor no other abnormality was revealed. The blood count was as follows: hematocrit = 28%, WBC = 10,000/μl, 15% blasts, 10% neutrophils, 30% lymphocytes, 40% large monocytes, 5% metamyelocytes and 60,000/μl platelets. The myelogram showed marked infiltration of the bone marrow by immature blasts, while the electrophoresis and immunoelectrophoresis did not differ from that of his previous admission. The diagnosis of AL was established. As cytochemical studies were, unfortunately, not performed the exact type of AL cannot be identified with certainty. The patient died 15 days following his second admission. The peripheral blood picture 24 h before death was: hematocrit = 25%, WBC = 190,000/μl of which 90% blasts, 2% neutrophils, 4% lymphocytes, 4% large mononuclears and 150,000/μl platelets.

Discussion

AL has been observed in a number of cases as a final event in patients suffering from MM.

The first well-documented case was reported in 1966 by *Osserman and Lawlor* [1] in a patient aged 44 who developed monomyelocytic leukemia, 48 months after following the diagnosis of MM for which he had received treatment with melfalan, urethane and irradiation. A good number of similar reports has followed [2–1]. This has led to the hypothesis that treatment may be responsible for the development of AL in patients suffering from MM [3].

The first case of MM which developed AL without any treatment was described by *Videback* [5] in 1971. *Tursz et al.* [20] reported another two similar cases of MM followed by AL in which no treatment for the myeloma was given. The present case is similar to those described by *Videback and Tursz*. We believe that in our case the infiltration of the bone marrow by numerous plasma cells, some of which were immature, together with monoclonal gammopathy leaves no doubt about the diagnosis of myeloma. It is regrettable that owing to lack of facilities at that time the study of the case was incomplete. In the cases described in the literature the AL was of the myelomonocytic type. In the present case the type of AL was not identified, as neither histochemical techniques were performed nor lysozyme was determined in the serum and the urine.

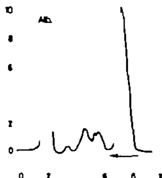


Fig. 1. Serum electrophoretic pattern showing monoclonal spike in the gamma region.

References

1 Osserman, E. F. and Lawlor, D. P.: Serum and urinary lysozyme (muramidase) in monocytic and monomyelocytic leukemia. J. exp. Med. *124*: 921–952 (1966).

2 Edwards, G A. and Zawadzki, Z. A., Extraosseous lesions in plasma cell myeloma. Am. J Med. 43 194–205 (1967)
3 Kyle, R. A., Pierre, R V and Bayrd, E. D., Multiple myeloma and acute myelomonocytic leukemia. Report of four cases possibly related to Melfalan. New Engl. J Med. 283 1121–1125 (1970)
4 Osserman, E. F., Monocytic and monomyelocytic leukemia with increases serum and urine lysozyme as a late complication in plasma cell myeloma. Br med. J I 327 (1971)
5 Videback, A., Unusual cases of myelomatosis. Br med. J II. 326 (1971)
6 Webb J A. W Bateman, C. J T and Davies, J D., Leukemia on myeloma. Br med. J Iv 231 (1971).
7 Scamps, R. A. O'Neill, B J and Newland, R. C. A case of multiple myeloma terminating with acute myelomonocytic leukemia. Med. J Aust. II. 1129 (1971)
8 Van Hove, W Hamers, J and Demeulenaere, L., Acute leukemia in myeloma. Lancet II. 570 (1973)
9 Khallell, M Keane, W M and Lee, G R. Sideroblastic anemia in multiple myeloma. A preleukemic change. Blood 41 17–25 (1973)
10 Rosner F and Grünwald, H Multiple myeloma terminating in acute leukemia Am. J Med. 57 927–939 (1974)
11 Low I P., Plovnlck, H. S., and Beddow D G Multiple myeloma, sideroblastic anemia and acute leukemia. New Engl J Med. 15 194 (1976).
12 Gonzalez, F Trujillo, J and Alexanian, R Acute leukemia in multiple myeloma. Ann. Intern. Med. 86 440–443 (1977).
13 Hooland, J F Epidemic acute leukemia. New Engl. J Med. 283 1165–1166 (1970)
14 Mills, R. C. Cornwell, G G and Mc Intyre O R., Remission of leukemia associated with multiple myeloma. New Engl. J Med. 285 920–921 (1971)
15 Cohen, S. L., Dodsworth, H., and Whitelaw A. G L., Leukemia on myeloma. Br med. J Iv 490 (1971).
16 Quirt, I. C., Hart, G D and Soots, M., Leukemia on myeloma. Br med. J I. 248 (1972).
17 Holt, J M. Robb-Smith, A. H. T Callender S. T., and Spiggs, A. L. Multiple myeloma. Development of alternative malignancy following successful chemotherapy Br J Hemat. 22. 633 (1972)
18 Riedler G F., Vorburger, E. und Maier C. Unreifzellige Leukosen und Myelom. Schweiz. med. Wschr 102 1592 (1972).
19 Taddini, L. and Schrader, W Concomitant myelomonocytic leukemia and multiple myeloma. Minerva Med. 55 446 (1972).
20 Turaz, T H., Flandrin, G Brouet, J-C., Briere, J and Seligmann, M., Simultaneous occurrence of acute myeloblastic leukemia and multiple myeloma without previous chemotherapy Br med. J II. 642–643 (1974)
21 Stavem, P and Harboe, M., Acute erythroleukemia in a patient treated with melfalan for the cold agglutinin syndrome. Scand. J Hematol. 8 375 (1971).

Received October 16, 1978
Accepted February 15 1979

George A. Kastanas, MD 6, Ipirou Street, Athens 103 (Greece)

Acta haemat. 62: 81–85 (1979)

Hyperviscosity Syndrome in a Patient with Plasma Cell Leukemia

Dan Douer, Avraham Weinberger, Meir Djaldetti, Jack Asherov, Israel Pick and Jack Pinkhas

Department of Medicine D, Section of Clinical Immunology,
Rogoff Wellcome Medical Research Institute, Beilinson Medical Center and
Department of Medicine B, Hasharon Hospital, Tel Aviv University Medical School, Petah Tikva

Key Words. Hyperviscosity syndrome · M-component · Plasma cell leukemia · Plasmapheresis

Abstract. A patient with plasma cell leukemia and IgG (K) M-component, who developed a hyperviscosity syndrome is reported. To our knowledge, this complication has not yet been reported in plasma cell leukemia.

The incidence of plasma cell leukemia (PCL) among multiple myeloma (MM) patients has been estimated at 1.6–2% [1–4]. It seems that PCL has the same incidence of immunoglobulin abnormalities as in MM, but both diseases differ in their clinical manifestations and course [4]. The hyperviscosity syndrome, known to frequently occur in macroglobulinemia [10], may also complicate the course of IgG and IgA MM [7]. We presently report a patient with PCL and the hyperviscosity syndrome; to our knowledge, a similar observation has not yet been described.

Case Report

A 62-year-old farmer was admitted to our department in April 1977, 6 weeks previously PCL was diagnosed in another hospital. The peripheral white blood cell count was 30,000/μl, of which 20% were immature plasma cells. The bone marrow aspiration biopsy revealed 70% plasma cells and IgG (monoclonal) was detected in the serum. Treatment, consisting of melphalan and prednisone, was followed by the disappearance of the plasma cells from the peripheral blood. Gradually low back pain and weakness appeared and the patient was referred to our department. Pallor and marked tenderness over the spinal column were noted. The liver and the spleen were palpable 2 cm below the costal margin, both firm and nontender. The erythrocyte sedimentation rate (Westergren) was 115 mm/h, the hemoglobin 6.9 g/dl, the white blood cell count 9,400/μl and the platelet count 97,000/μl. Plasma cells were not detected in the peripheral blood smear. Bence Jones protein was not detected. The bone marrow aspiration biopsy revealed a massive infiltration of immature plasma cells. The skeletal X-ray survey showed multiple osteolytic lesions. The total serum protein and immunoglobulin levels are shown in table I. Cellulose acetate electrophoresis of the serum revealed an M-component in the slow γ-region which was identified by immunoelectrophoresis as IgG with κ light chains. Treatment



Fig. 1 Eyeground examination. Note the retinal hemorrhages and the engorged veins.

consisted of intravenously dministered cyclophosphamide, prednisone and blood transfusions. The patient s condition improved, the bone pai almost disappeared and he was discharged whil on oral cyclophosphamide and prednisone.

6 months later (October 1977), he was readmitted because of recurrence of bone pain, confusion, mucosal bleeding and blurring of vision. The eyeground examination revealed multiple retinal hemorrhages and sausage shaped retinal veins (*fig. 1*) The liver and the spleen were palpable 5 cm below the costal margin, both firm and nontender The hemoglobin was 7.3 g/dl, the whit blood cell count 14,600/ l with 32* mature and immature plasma cells (fig. 2) and the platelet count 92,000/ l The blood urea itrogen was 67 mg/dl the serum calcium 9.5 mg/dl and the relative viscosity of the serum at 37 °C was 5.8 cP (normal < 3 cP) Quantitation of total serum proteins, immunoglobulins and IgG (K) M-component are given in table I Electron microscopic examination of the peripheral blood revealed plasma cells in different stages of maturation (fig. 3). Treatment consisted of prednisone, cyclophosphamide, repeated plasmaphereses and transfusions of packed red blood cells. The peripheral white blood cell count rose to 97,000/ l with 78* plasma cells, and the patient died 3 weeks later

Table I. Serum protein and immunoglobulin concentrations

	April 1974	October 1974	Normal range
Total protein, g/dl	11.3	13.2	5–8
Albumin, g/dl	3.15	4.77	3–5
IgG M-component, mg/dl	6,000	6,600	
IgG M-component, mg/dl	trace	trace	
IgA, mg/dl	105	0	260 ± 65
IgM mg/dl	25	0	130 ± 35

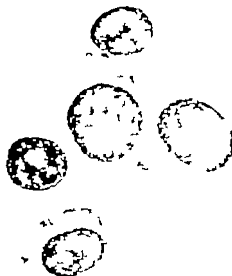


Fig. 2. Peripheral blood smear. Note many plasma cells. May-Grünwald-Giemsa. × 1,200

On autopsy, diffuse plasma cell infiltration in the lungs, bones, lymph nodes and the gastrointestinal tract as well as multifocal hemorrhages were found.

Discussion

PCL and MM are considered to be different clinical manifestations of the common group of plasma cell dyscrasias [7]. In comparison to MM, patients with PCL tend to have a higher incidence of hepatosplenomegaly, marked leukocytosis and diffuse bleeding phenomena, multiple organ involvement and a more rapid and fatal clinical course, usually unresponsive to chemotherapy [4, 6, 7]. Three phases were apparent in the clinical course of the presently reported patient. The first phase was consistent with PCL and a 6-week clinical improvement was achieved by treatment with melphalan and prednisone. The second phase was marked by the finding of osteolytic lesions; clinically overt MM was diagnosed and the patient responded to cyclophosphamide-prednisone treatment. The third phase was characterized by the reappearance of PCL, hyperviscosity syndrome and lack of response to treatment.

The hyperviscosity syndrome complicates macroglobulinemia in 33–72% of the patients [10], while in IgG MM this syndrome was described in 4–8% of the patients only [8, 9]. Recently this syndrome has been diagnosed with increasing frequency in IgA MM [12]. To the best of our knowledge this syndrome has not yet been described in PCL. *Pruzansky et al.* [7] who reviewed 57 patients with PCL and *Kyle et al.* [4] who described 17 additional patients did not mention the occurrence of the hyperviscosity syndrome in PCL.

In macroglobulinemia, the hyperviscosity syndrome is due to the large asymmetrical IgM molecule [2]. In IgG and IgA MM this syndrome has been ascribed to several mechanisms, such as high M-component



Fig. 3 A mature plasma cell in the peripheral blood. × 9,830.

concentration, polymerization of monomers to high molecular weight components and to asymmetry of the myeloma protein [2, 3 5 7] We cannot state with certainty the cause of the hyperviscosity in our patient, since we could not demonstrate the existence of monomer polymerization. However the IgG M-component concentration in our patient was 6 g/dl and, as shown by *Pruzansky et al* [7] in almost all patients with IgG MM and the hyperviscosity syndrome, the M-component usually exceeds 5 g/dl. Moreover the hyperviscosity syndrome was apparent in 22% of patients with MM in whom the IgG M component was present at a concentration above 5 g/dl.

It was presumed in the past that the immature leukemic plasma cells were unable to synthesize immunoglobulins [7 11] However more recent studies, utilizing improved methods for the detection of abnormal proteins have shown that the abnormal serum proteins in PCL occur with the same frequency and are similar to those found in MM [4 7] Therefore, it seems reasonable to assume that the hyperviscosity syndrome can occur in PCL under the same circumstances as in MM and it should be investigated especially when high M-component levels are observed Plasmapheresis may cause remarkable improvement of the symptoms attributable to increased serum viscosity in patients with MM [2] and this policy should be adopted in PCL patients with the hyperviscosity syndrome The lack of response to treatment in the final stage of the disease may reflect the failure of the cytotoxic agents to suppress the clone-producing M-component and may eventually explain the ineffectiveness of the repeated plasmaphereses.

References

1 Bichel, J Effersoe, P., Gormsen, H and Harboe, N.. Leukemic myelomatosis (plasma cell

leukemia). A review with report of four cases Acta radiol. *37* 196–207 (1952).
2 Bloch, K. J and Maki, D. G Hyperviscosity syndromes associated with immunoglobulin abnormalities. Semin. Hematol. *10* 113–124 (1973).
3 Capra, J D. and Kunkel, H G. Aggregation of IgG proteins: relevance to the hyperviscosity syndrome. J clin. Invest. *49* 610–621 (1970).
4 Kyle, R. A., Maldonado, J E., and Bayrd, E. D Plasma cell leukemia. Report on 17 cases. Archs intern. Med. *133* 813–818 (1974).
5 Mackenzie, M R., Fudenberg, H H., and O'Reilly R. A. The hyperviscosity syndrome. I. In IgG myeloma. The role of protein concentration and shape. J clin. Invest. *49* 15–20 (1970).
6 Pollack, A Rachmilewitz, D and Zlotnick, A. Plasma cell leukemia Unassembled light and heavy chains in the urine Archs intern. Med. *134* 131 134 (1974).
7 Pruzansky W Platts, M. E., and Ogryzlo, M A. Leukemic form of immunocytic dyscrasia (Plasma cell leukemia) Am. J Med *47* 60–74 (1969)
8 Pruzansky W and Watt, J G.: Serum viscosity and hyperviscosity in IgG multiple myeloma. Ann. intern. Med. *77* 853–860 (1972).
9 Schur P H., Kyle, R. A., Bloch, K. J *et al.* IgG subclasses relationship to clinical aspects of multiple myeloma and frequency of distribution among M-components. Scand. J Hematol *12*. 60–68 (1974).
10 Somer T Hyperviscosity syndrome and plasma cell dyscrasia. Adv Microcirc vol. 6, pp. 1 55 (Karger Basel 1975).
11 Thorling, E. B., Leukemic myelomatosis (plasma cell leukemia). Acta haemat. *28* 222–229 (1962).
12 Virella, G Preto, R. V and Graca, F Polymerized monoclonal IgA in two patients with myelomatosis and hyperviscosity syndrome. Br J Haemat. *30* 479–487 (1975).

Received: January 10, 1979
Accepted. March 1 1979

Prof. Jack Pinkhas, MD, Department of
Medicine D Beilinson Medical Center
Petah Tikva (Israel)

Acta haemat. 62 86–89 (1979)

Platelet Transfusion Requirements of Children with Newly Diagnosed Lymphoblastic Leukaemia

S J Ilett and J S Lilleyman

Department of Haematology The Children s Hospital Western Bank, Sheffield

Key Words. *Lymphoblastic leukaemia Platelet transfusions*

Abstract. The platelet transfusion requirements of 70 unselected children with lymphoblastic leukaemia were studied from the time of diagnosis to the achievement of complete remission, or to death if no remission was obtained. Platelets were not transfused unless clinically significant bleeding occurred in association with a platelet count of less than 20 0 × 10⁹/l and on this basis only 31 transfusions were given to 11 (15%) patients for 17 episodes of apparent or presumed bleeding. No deaths occurred due to haemorrhage, and 67 (95%) patients achieved complete remission These findings suggest that such children do not need prophylactic platelet transfusions, which are commonly given despite their inherent risks and sequelae.

Introduction

It is widely recommended that platelet transfusions should be given prophylactically to patients with acute leukaemia and consequent thrombocytopenia [1 2, 4 5] and randomised studies have indicated a reduction in bleeding in these patients in the case of both adults [1] and children [4]

Such prophylactic platelet replacement is not always of obvious value however [7] and a recent review has pointed out some of the problems following repeated platelet transfusions suggesting a need for critical re-examination of the indications for them [8]

For this reason we have reviewed 5 years experience of a sparing approach to platelet replacement (both in application and dose) in a group of patients where inadequate supportive therapy could mar a good prognosis – children undergoing the early stages of treatment for newly diagnosed acute lymphoblastic leukaemia (ALL)

Patients and Methods

The patient described were all under 14 years old a d were consecutive new cases of ALL diagnosed and treated at the Sh ffield Children s Hospit l. The diagnosis wa established on conventional clinical, morphological nd cytochemical

Table I. Patients dying without achieving remission in ALL

Patient	Age and sex	Leucocyte count at diagnosis $10^9/l$	Time from diagnosis to death months	Platelet transfusions given	Bleeding episodes treated with platelets	Primary cause of death
A. C.	7 M	6.5	1	6	1	septicaemia
M. M	9 M	412.0	9	4	2	resistant bone marrow disease
M. B	3 F	45.0	1	1	1	septicaemia

One transfusion given prior to lumbar puncture

grounds with the addition, latterly of membrane marker studies. All patients were treated initially with vincristine and prednisolone, and some had, in addition, asparaginase, cyclophosphamide and cytosine arabinoside during the remission induction period.

Platelet transfusions consisted of platelet concentrate prepared by doubl spin technique using fresh blood collected into acid citrat dextrose and suspending the platelets in 20–30 ml of supernatant plasma. Occasionally platelet were given as single-spin platelet-rich plasma for expediency wher volume was not problem. The dose of platelets given, as one transfusion on daily basis, varied from 3 to 10 donor units equivalent per square meter of body surface area ith the lower dose being the more common, and no attempt was made to achieve measurable rise in platelet count

The indication for platelet replacement used throughout the period under review was the presence of clinically significant bleeding associated ith platelet count of less than 20.0 $10^9/l$. Significant bleeding was defined as obvious or presumed haemorrhage, internal or external, including (fresh) fundal haemorrhages but excluding simple purpura and ecchymoses

A therapy was individually modified in patients with relapsed and subsequently refractory disease, each child w considered only from the time of diagnosis to the achievement f first complete remission (CR) or death if no CR as obtained. CR was defined as good health associated ith normally cellular bone marrow supporting normal peripheral blood and h no evidence of acti leukaemia.

Results

During the 5 years to January 1978, 70 children were diagnosed as suffering from ALL.

3 (4 /) died without achieving remission and details of their primary cause of death and survival time are shown in table I together with their platelet transfusion record, and leucocyte count at diagnosis.

59 (84 /) achieved remission without platelet transfusions being given. 34 of these at some time had a platelet count of $< 20.0 \times 10^9/l$. These 34 patients spent, by addition, a total of 255 days with $< 20.0 \times 10^9/l$ platelets but had no significant bleeding.

8 (11) patients who achieved CR received platelets prior to this event. Details of these children and of the relevant bleeding episodes together with the presence of any complicating factor (such as infection) at the time platelets were given are shown in table II. From this it will be seen that only 3 patients bled spontaneously apparently unassociated with infection, although 2 patients were given platelet cover for lumbar punctures while profoundly thrombocytopenic. From the total of 11 patients who received platelets, 5 (46) had leucocyte

Table II Patients receiving platelet transfusions during successful remission induction for ALL

Patient	Age and sex	Leucocyte count at diagnosis $\times 10^9/l$	Reason for platelet transfusion	Platelet transfusions per episode	Complicating factor evident	Time from diagnosis to CR days
J W	9 M	105.0	epistaxis and retinal haemorrhages	1	–	35
K B	1 F	6 0	(1) melaena	2	pneumonia	33
			(2) lumbar puncture	1		
S G	3 F	14 0	haematuria	2	urinary tract infection	36
R. C.	12 M	105 0	aspiration of haemopericardium	1	–	24
I W	4 M	17 4	(1) peri-orbital haematoma	2		
			(2) haematuria	2	jaundice and fever	46
			(3) haematuria	2		
K H	4 M	6.2	epistaxis	2	–	33
A B.1	2 M	70.0	(1) epistaxis	1		
			(2) lumbar puncture	1	septicaemia	46
A B.2	5 M	4.3	(1) epistaxis	2		
			(2) epistaxis	1	septicaemia	30

counts at diagnosis over 20 0 $\times 10^9/l$, but this was also the case in 13 (38%) of the 34 patients achieving remission uneventfully de spite thrombocytopenia. Likewise there was no treatment difference evident between the various subgroups.

Discussion

Since platelet transfusions have become readily available, the repeated infusion of homologous ABO compatible non-HLA matched platelets into patients with acute leukaemia and consequent thrombocytopen ia has been widely recommended on the ba sis of prevention rather than the arrest, of haemorrhage [1 2, 4 5]

Despite some evidence that such therapy reduces the incidence of bleeding [1 4] this has been questioned [7] and no confident claim has been made that a beneficial effect is apparent on overall survival Also in addition to the immediate hazards associated with the transfusion of a homologous blood component, there are later problems, of which the most significant is allosensitisation particularly as the recipient is exposed to a relatively large number of donors. Apart from the very high incidence of con sequent lack of haemostatic effect of platelet transfusions [8] a perhaps equally serious problem is the disadvantage patients with such tissue antibodies might have as potential bone marrow recipients if transplantation is attempted at a later stage [3] although this may be more relevant to pa tients with aplastic anaemia

The conservative policy of platelet replacement at our centre has not changed with improved platelet supplies, as no good evidence that this is necessary has emerged. In this 5-year review of 70 patients undergoing first remission induction therapy

no deaths due to bleeding occurred, which supports the claim that sparing platelets does not increase mortality despite this not being a controlled study It is possible that *morbidity* due to bleeding could have been slightly reduced but arguably not to a degree worth the price of allosensitization.

Children with newly diagnosed ALL have the best outlook of all patients with acute leukaemia, and therefore supportive therapy should be optimal. Several years ago, when policy for platelet transfusion was dictated largely by a restricted supply *Simone* [6] noted their relatively modest re quirements in this respect. We would not only endorse this, but also suggest that provided platelets can be obtained quickly there is little to gain and more to lose by giving them to children with ALL who have no significant bleeding. The only exception we would make to this is the elective cover of procedures likely to precipitate bleeding when thrombocytopenia is present, such as lumbar punctures or minor surgery

We have made no comment on patients with other types or stages of leukaemia as they would form a very heterogeneous group about which it would be difficult to generalise The main purpose of this report is to identify a relatively homogenous group of thrombocytopenic patients in whom platelet transfusion in the absence of bleeding (or a bleed provoking procedure) is not in dicated.

Acknowledgements

We wish to thank *Dr W Wagstaff* and the staff of the Sheffield Regional Blood Transfusion Service for their help and supplies of platelets. J S. L. is in receipt of grant from the Leukaemia Research Fund of Great Britain.

References

1 Higby D J Cohen, E Holland, J F and Sinks, L. The prophylactic treatment of thrombocytopenic leukaemic patients with platelets: doubl blind study Transfusion *14* 440–446 (1974).

Hoak, J C. and Koepke, J A. Platelet transfusions. Clin Haematol *5* 69–79 (1976).

3 Kay A. B. Some complications associated with the administration of blood and blood products. Clin. Haematol. *5* 165–181 (1976).

4 Murphy S., Kock, P A., and Evans, A. E. Randomised trial of prophylactic vs. therapeutic platelet transfusion in childhood acute leukaemia. Abstract. Clin Res *24* 379A (1976)

5 Schiffer C. A. Some aspects of recent advances in the use of blood cell components Br J Haemat. *39* 289–294 (1978).

6 Simone, J V Use of fresh blood components during intensive combination therapy of childhood leukaemia. Cancer *28*. 562 565 (1971).

7 Solomon, J Bofenkamp, T Fahey J L., Chillar R. K., and Beutler E. Platelet prophylaxis in acute non-lymphoblastic leukaemia. Lancet 267 (1978).

8 Van Eys, J Thomas, D., and Olivos, B., Platelet use in paediatric oncology: review of 393 transfusions. Transfusion *18* 169–173 (1978).

Received. January 22, 1979
Accepted: March 9 1979

Dr J S Lilleyman, Head, Department of Haematology Sheffield Children Hospital, Western Bank, Sheffield S10 2TH (England)

Acta haemat. 62 90–93 (1979)

Myeloproliferative Disease of Childhood Associated with a Trisomy 21 Clone

E Tabachnik R Garty R Zaizov and J Chemke

Department of Pediatrics B, Clinical Genetics Unit, Kaplan Hospital
(affiliated to the Hebrew University and Hadassah School of Medicine), Rehovot,
Department of Pediatrics, Beilinson Hospital, Petah Tikva, and Sackler School of Medicine,
Tel Aviv University Tel Aviv

Key Words. Myeloproliferative disease Chromosome abnormality Trisomy 21 Chronic myeloid leukemia

Abstract. *Myeloproliferative disease of childhood is frequently associated with chromosomal anomalies, usually of the C group Clinical features are similar to those of the juvenile type of chronic myeloid leukemia.* A child with this disease is described. Marked myeloid proliferation anemia, thrombocytopenia and hepatosplenomegaly were present leukocyte alkaline phosphatase and fetal hemoglobin were moderately elevated Chromosome analysis of bone marrow cells revealed a mosaicism 47,XX,+21/46,XX. Down s syndrome was ruled out by the child s normal phenotype and dermatoglyphic analysis. The cytogenetic finding is probably evidence for the clonal origin of the trisomy 21 cell line

Introduction

Myeloproliferative diseases, characterized by a primary disturbance of granulocyte proliferation, are uncommon in childhood. Chronic myeloid leukemia (CML) represents only 2–5% of childhood leukemia [1 2, 4 8 9] and is considered to exist in two main forms one, indistinguishable from the classic 'Ph -positive CML seen in adults, is known as the adult type of CML the other so-called *juvenile myeloid leukemia* has strikingly different clinical laboratory and cytogenetic characteristics [6] In addition to these, there have been many reports, in recent years, of myeloproliferative disorders in childhood, associated with chromosomal anomalies, usually of the C group [7 12]

We report a case of a myeloproliferative disorder in a 7 year-old child associated with a trisomy 21 clone in bone marrow cells.

Case Report

J J a female child of Jewish Moroccan descent, was first referred to the Department of Pediatrics at age 18 months because of fever and hepatosplenomegaly A peripheral blood smear as

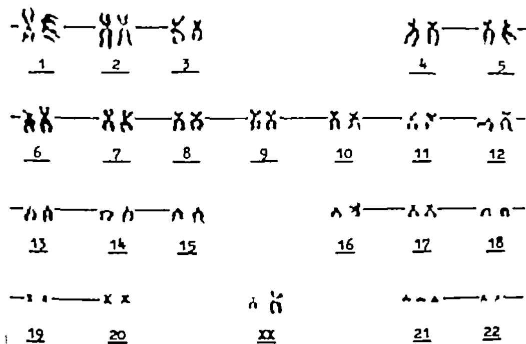


Fig. 1. Karyotype 47,XX,+21 in bone marrow (G banding)

well as bone marrow findings supported the diagnosis of infectious mononucleosis. A repeat blood smear was normal 1 month later and the child remained well until the age of 7 years when she was readmitted because of transient swelling of the left knee. Peripheral blood smears as well as roentgenological examination of the knee were normal. 8 months later the child was once again hospitalized, this time because of multiple ecchymoses which had appeared during the month prior to admission.

The patient was well developed for her age with normal female phenotype. She was pale and looked ill. Pertinent findings on physical examination included ecchymoses of varying sizes which were scattered over most parts of the body. Petechiae were noted on the buccal mucosa. Liver and spleen were both palpable 7 cm below the costal margins. Enlarged inguinal and axillary lymph nodes were palpated. No skeletal or neurological abnormalities were demonstrated. Dermatoglyphic analysis was normal.

Pertinent laboratory findings on admission were as follows: hemoglobin 6.4 gm%, hematocrit 18%, reticulocytes 17%, leukocytes 68,000/l consisting of myeloblasts 1%, metamyelocytes 4%, stabs 47%, polymorphs 31%, lymphocytes 6% and monocytes 9%. Thrombocytes 23,000/l. Marked anisocytosis and occasional basophilic granulations were noted on the peripheral blood smears. Bone marrow examination revealed marked proliferation of the myeloid series, ranging from myeloblasts to mature polymorphs, and a striking absence of megakaryocytes. The myeloid/erythroid ratio was 10:1. Leukocyte alkaline phosphatase (LAP) score 326 (Kaplow, normal 50–150). Fetal hemoglobin 11.4% with normal hemoglobin A_2; vitamin B_{12} was markedly elevated (5,687 pg/ml). Blood chemistry was normal. Immunological studies were suggestive of cellular immune deficiency.

Chromosome analysis was performed on bone marrow cells, cultured for 24 h at 37 °C in F 10 medium supplemented with 15% fetal calf serum without phytohemagglutinin. Mitoses were arrest-

ed with Colcemid. Giemsa banding patterns were obtained after trypsin digestion following a modification of *Seabright's* [11] method. Thirty five cells were analyzed. In 31 cells (88%), an additional chromosome No 21 was observed (47,XX,+21) whereas the karyotype was normal female in 4 cells (fig. 1) Chromosome analysis from a phytohemagglutinin-stimulated peripheral blood lymphocyte culture was unsuccessful. A skin biopsy did not yield fibroblasts.

After admission, the white cell count increased progressively reaching 300,000/μl within 2 weeks, followed by a spontaneous short lived decrease to 14,000/μl. Occasional myeloblasts were noted in peripheral blood smears. Repeated episodes of hemolysis as well as persistent thrombocytopenia necessitated several red blood cell and thrombocyte transfusion. 5 weeks after admission, because of continued thrombocytopenia and progressively increasing leukocyte counts, chemotherapy with Busulfan and steroids was initiated but without response. A massive intracranial hemorrhage was the terminal cause of death 3 months after the initial diagnosis. *Post mortem* examination was refused.

Discussion

Increased numbers of myeloid cells at all stages of maturation together with anemia, thrombocytopenia and hepatosplenomegaly are consistent with the diagnosis of chronic myeloproliferative disorder Adult-type CML was not considered since a Philadelphia chromosome was not demonstrated and features such as thrombocytopenia and the high levels of LAP are against this diagnosis [6 9] On the other hand juvenile type CML was thought unlikely because of the elevated LAP values and also because the fetal hemoglobin values were not nearly as high as expected [6 7] as opposed to the patient described by *Cáp et al* [3]

In our opinion this patient should be classified together with the cases of myeloproliferative disease associated with chromosomal abnormalities as described by *Humbert et al* [7] who were the first to focus attention on the association between this condition and a missing C-group chromosome. Since then there have been a spate of reports of chromosomal loss or gain in myeloproliferative disease mainly of the C group [5 7] their clinical features being similar to those of the juvenile-type CML. Most children die within 1 year of the diagnosis [7] LAP levels are usually elevated as was the case in our patient.

The trisomy 21 cell line was obviously an abnormal clone since only 88% of the examined bone marrow cells demonstrated an extra chromosome. A trisomy 21 clone is an uncommon event, and abnormalities of chromosome 21 have been described mainly in acute leukemia [13] Nonneoplastic clones appear to be very rare in hematopoietic tissues, even though clonal evolution may long precede the neoplastic transformation [10] In the present case, the finding of mosaicism 47,XX,+21/46,XX in bone marrow cells can be viewed as evidence for the clonal origin of the trisomy 21 cell line. The diagnosis of Down's syndrome can be ruled out since the child's phenotype and dermatoglyphic analysis were normal and chromosomal constitution was normal in 12% of the bone marrow cells examined.

To the best of our knowledge this type of chromosomal aneuploidy has not previously been described in childhood chronic myeloproliferative disorder The present case emphasizes the need for systematic cytogenetic investigations in this disease.

References

1 Barrett, O., Conrad, M., and Crosby W Chronic granulocytic leukemia i childhood. Am. J med. Sci 240 587–593 (1960).

2 Bernard, J. Seligmann, M., and Acar, J. Chronic myeloid leukemia of children – Study of 20 cases. Archs. fr. Pediat. *19* 881–894 (1962).
3 Cáp, J. Izakovič, V. und Vojtaššák, J.: Juvenile Form der chronischen myeloischen Leukämie bei einem 3½ jährigen Knaben mit Mosaikvorkommen der Trisomie 21 ohne Merkmale eines Down-Syndroms. Mschr. Kinderheilk. *125* 740–743 (1977).
4 Cooke, J. Chronic myelocytic leukemia in children. J. Pediat. *42* 537–546 (1953).
5 Gahrton, G. Lindsten, J. and Zech, L.. Involvement of chromosome 8, 9 19 and 22 in Ph positive and Ph₁ negative chronic myelocytic leukemia in the chronic or blastic stage. Acta med. Scand. *196*, 355–360 (1974).
6 Hardisty, R. M., Speed, D. E., and Till, M.. Granulocytic leukemia in childhood. Br. J. Haemat. *10*: 551–566 (1964).
7 Humbert, J. R., Hathaway, W. E., Robinson, A., Peakman, D. C., and Githens, J. H.: Preleukemia in children with missing bone marrow C chromosome and myeloproliferative disorder. *Br. J. Haemat. 21* 705–716 (1971).
8 Lightwood, R. Barrie, H., and Butler, N.: Observations on 100 cases of leukemia in childhood. Br. med. J. *I.* 747–752 (1960).
9 Reisman, L. and Trujillo, J. Chronic granulocytic leukemia of childhood. J. Pediat. *62*: 710–723 (1963).
10 Nowell, P. C.. Chromosome changes and the clonal evolution of cancer. Chromosomes and cancer pp. 267–285 (*Wiley, New York 1974*).
11 Seabright, M.. Rapid banding technique for human chromosomes. Lancet *ii*. 971–972 (1971).
12 Teasdale, J. M., Worth, A. J. and Corey, M. J. A missing group C chromosome in the bone marrow cells of three children with myeloproliferative disease. Cancer *25* 1468–1477 (1970).
13 Yamada, K. and Furusawa, S.. Preferential involvement of chromosomes No. 8 and No. 21 in acute leukemia and preleukemia. Blood *47* 679–686 (1976).

Received: November 13, 1978
Accepted: February 16, 1979

Dr. Juan Chemke, Director
Clinical Genetics Unit, Kaplan Hospital,
Rehovot (Israel)

ed with Colcemid. Giemsa banding patterns were obtained after trypsin digestion following a modification of *Seabright's* [11] method. Thirty five cells were analyzed. In 31 cells (88%), an additional chromosome No 21 was observed (47 XX,+21), whereas the karyotype was normal female in 4 cells (fig. 1) Chromosome analysis from a phytohemagglutinin-stimulated peripheral blood lymphocyte culture was unsuccessful. A skin biopsy did not yield fibroblasts.

After admission, the white cell count increased progressively reaching 300,000/μl within 2 weeks, followed by a spontaneous short-lived decrease to 14,000/μl Occasional myeloblasts were noted in peripheral blood smears. Repeated episodes of hemolysis as well as persistent thrombocytopenia necessitated several red blood cell and thrombocyte transfusion. 5 weeks after admission, because of continued thrombocytopenia and progressively increasing leukocyte counts, chemotherapy with Busulfan and steroids was initiated but without response. A massive intracranial hemorrhage was the terminal cause of death 3 months after the initial diagnosis. *Post mortem* examination was refused.

Discussion

Increased numbers of myeloid cells at all stages of maturation together with anemia, thrombocytopenia and hepatosplenomegaly are consistent with the diagnosis of chronic myeloproliferative disorder Adult type CML was not considered since a Philadelphia chromosome was not demonstrated and features such as thrombocytopenia and the high levels of LAP are against this diagnosis [6, 9] On the other hand, juvenile-type CML was thought unlikely because of the elevated LAP values and also because the fetal hemoglobin values were not nearly as high as expected [6, 7] as opposed to the patient described by *Čáp et al* [3]

In our opinion this patient should be classified together with the cases of myeloproliferative disease associated with chromosomal abnormalities as described by *Humbert et al* [7] who were the first to focus attention on the association between this condition and a missing C-group chromosome. Since then there have been a spate of reports of chromosomal loss or gain in myeloproliferative disease mainly of the C group [5 7] their clinical features being similar to those of the juvenile type CML. Most children die within 1 year of the diagnosis [7] LAP levels are usually elevated as was the case in our patient.

The trisomy 21 cell line was obviously an abnormal clone since only 88% of the examined bone marrow cells demonstrated an extra chromosome. A trisomy 21 clone is an uncommon event, and abnormalities of chromosome 21 have been described mainly in acute leukemia [13] Nonneoplastic clones appear to be very rare in hematopoietic tissues, even though clonal evolution may long precede the neoplastic transformation [10] In the present case, the finding of mosaicism 47,XX,+21/46,XX in bone marrow cells can be viewed as evidence for the clonal origin of the trisomy 21 cell line The diagnosis of Down's syndrome can be ruled out since the child's phenotype and dermatoglyphic analysis were normal and chromosomal constitution was normal in 12% of the bone marrow cells examined.

To the best of our knowledge this type of chromosomal aneuploidy has not previously been described in childhood chronic myeloproliferative disorder The present case emphasizes the need for systematic cytogenetic investigations in this disease.

References

1 Barrett, O Conrad, M., and Crosby W Chronic granulocytic leukemia in childhood. Am. J med. Sci *240* 587–593 (1960).

2 Bernard, J.; Seligman, M., and Acar, J: Chronic myeloid leukemia of children. Study of 20 cases. Archs. fr. Pediat. *19:* 881–894 (1962).
3 Cip, J; Izakovič, V. und Vojtaššák, J: Juvenile Form der chronischen myeloischen Leukämie bei einem 3 $^{1}/_{12}$ jährigen Knaben mit Mosaikvorkommen der Trisomie 21 ohne Merkmale eines Down-Syndroms. Mschr. Kinderheilk. *125:* 740–743 (1977).
4 Cooke, J: Chronic myelocytic leukemia in children. J. Pediat. *42:* 537–546 (1953).
5 Gahrton, G; Lindsten, J, and Zech, L: Involvement of chromosome 8, 9, 19 and 22 in Ph₁ positive and Ph₁ negative chronic myelocytic leukemia in the chronic or blastic stage. Acta med. Scand. *196:* 355–360 (1974).
6 Hardisty, R. M.; Speed, D. E., and Till, M: Granulocytic leukemia in childhood. Br. J. Haemat. *10:* 551–566 (1964).
7 Humbert, J. R.; Hathaway, W. E.; Robinson, A.; Peakman, D. C., and Githens, J. H: Preleukemia in children with a missing bone marrow C chromosome and a myeloproliferative disorder. Br. J. Haemat. *21:* 705–716 (1971).
8 Lightwood, R.; Barrie, H., and Butler, N: Observations on 100 cases of leukemia in childhood. Br. med. J. *i:* 747–752 (1960).
9 Reisman, L. and Trujillo, J: Chronic granulocytic leukemia of childhood. J. Pediat. *62:* 710–723 (1963).
10 Nowell, P. C.: Chromosome changes and the clonal evolution of cancer. Chromosomes and cancer pp. 267–285 (Wiley, New York 1974).
11 Seabright, M.: Rapid banding technique for human chromosomes. Lancet *ii:* 971–972 (1971).
12 Teasdale, J. M.; Worth, A. J., and Corey, M. J: A missing group C chromosome in the bone marrow cells of three children with myeloproliferative disease. Cancer *25:* 1468–1477 (1970).
13 Yamada, K. and Furusawa, S.: Preferential involvement of chromosomes No. 8 and No. 21 in acute leukemia and preleukemia. Blood *47:* 679–686 (1976).

Received: November 13, 1978
Accepted: February 16, 1979

Dr. Juan Chemke, Director
Clinical Genetics Unit, Kaplan Hospital,
Rehovot (Israel)

Acta haemat. 62 94–99 (1979)

Endogenous Erythroid Colony Formation by Peripheral Blood Mononuclear Cells from Patients with Myelofibrosis and Polycythemia Vera[1,2]

John D Lutton and Richard D Levere

Department of Medicine, New York Medical College, Valhalla, N Y

Key Words. Endogenous Erythroid colonies Myelofibrosis Polycythemia vera

Abstract. Peripheral blood mononuclear cells from patients with polycythemia vera or myelofibrosis with myeloid metaplasia were studied for their erythroid colony growth characteristics in plasma clot cultures. In both diseases, erythroid colonies formed early in culture in the absence of added erythropoietin (endogenous colonies) In no instance did early endogenous colony formation occur with peripheral blood cells from normals or patients with secondary polycythemia. A normal response to erythropoietin was observed with both control and patients peripheral blood cells. Spleen mononuclear cells obtained from one patient with myelofibrosis also produced endogenous colonies and showed a response to erythropoietin. This study suggests that culture of peripheral blood mononuclear cells might serve as a useful tool in discriminating polycythemia vera from secondary polycythemia.

Introduction

Myelofibrosis with myeloid metaplasia is a myeloproliferative disease characterized by an abnormal growth of hematopoietic precursors with varying degrees of fibrosis of the marrow Polycythemia vera is characterized by overproduction of several hematopoietic cell lines with red cell series being most prominent [5] It is often associated with myeloid metaplasia and may progress to myelofibrosis. The development of *in vitro* cell culture techniques has made it possible to assess the erythroid growth potential of bone marrow and peripheral blood [4 14] Colonies of erythropoietic cells are thought to originate from single stem cells and the capacity of such colonies to proliferate and differentiate under hormonal control serves as a useful tool to investigate the nature of the stem cells [1] Recently *Prchal et al* [13] have presented data suggesting a clonal origin of polycythemia vera

[1] Part of this data was presented at the Midwest Section of the American Federation for Clinical Research, November 3, 1977 Chicago, Ill.

[2] This work was supported by National Institutes of Health grant AM 21682.

and in other investigations have demonstrated that bone marrow cells from polycythemia vera patients grow in a unique way when compared to those of normals or secondary polycythemia patients [12]. *Zanjani et al.* [16] have demonstrated that this unique growth is characterized by the *in vitro* development of erythroid colonies in the absence of exogenous erythropoietin. Using a different technique *Horland et al.* [9] reported endogenous colony growth from the peripheral blood of 1 patient with myelofibrosis.

In the present study we wish to extend the above information and report the unusual *in vitro* behavior of mononuclear cells obtained from peripheral blood of patients with idiopathic myelofibrosis associated with myeloid metaplasia as well as patients with polycythemia vera. The data indicate that in both disorders, plasma clot cultures of peripheral blood mononuclear cells give rise to endogenous erythroid colonies in the absence of added erythropoietin.

Materials and Methods

Samples (20 ml) of normal or patient's peripheral blood were drawn in heparinized sterile tubes. Peripheral blood mononuclear cells were separated from erythrocytes by using the Ficoll-Hypaque density centrifugation technique of *Boyum* [2]. Briefly, heparinized whole blood is diluted with Hanks' balanced salt solution (HBSS) (Ca^{++}–Mg^{++} free) (Grand Island Biological Co.), carefully layered on Ficoll-Hypaque and centrifuged at 450 g for 30 min. The mononuclear cell layer is then removed, washed in HBSS, counted and resuspended to the desired cell concentration in minimal essential medium containing 2% fetal calf serum. Cell viability was routinely checked with trypan blue. Cell culture was done using the plasma clot culture technique of *Stephenson et al.* [14] as modified for growth of peripheral blood by *Clarke and Housman* [4]. Culture medium (NCTC 109) and other supplemental components were obtained from Grand Island Biological Co. The erythropoietin (Epo) used in these experiments was human urinary erythropoietin (lot H-12 TaLSL, US National Institutes of Health) and was added at concentrations of 0.25–4 U/ml of culture. Cultures were incubated in an atmosphere of high humidity and 4% CO_2 in air at 37 °C 4–14 days after which the clots were removed, squashed, fixed in glutaraldehyde and stained with *benzidine-hematoxylin*. Only erythroid colonies containing eight or more benzidine-positive cells were counted. This was done since the criteria for scoring human erythroid colonies and bursts have not been clearly established [10].

A clinical summary of patients with myelofibrosis and polycythemia is represented in table I. Nucleated red blood cells were present in the peripheral blood of all of the patients with myelofibrosis associated with myeloid metaplasia. 2 of the patients (AJ and AB) had a history of polycythemia vera which recently transformed into myelofibrosis with myeloid metaplasia. The diagnosis of polycythemia vera was based on criteria adopted by the National Polycythemia Vera Study Group.

Results

Erythroid colony counts from experiments using peripheral blood mononuclear cells obtained from 6 normals (A–F) and 2 patients with secondary polycythemia (ID NS) are presented in table II. When incubated with 2 IU Epo/ml, colony counts ranged from 0–36 erythroid colonies/10^6 cells at 10 days and 40–125 colonies/10^5 cells after 14 days. No erythroid colonies were observed after 5–7 days of incubation with Epo and in no instance were colonies observed when Epo was omitted from the cultures.

The numbers of erythroid colonies grown from peripheral blood mononuclear cells from 6 patients with myelofibrosis are shown in table III. In contrast to controls,

Table I Clinical summary of patients with myelofibrosis and polycythemia

Patient	Therapy	HC+%	WBC/Cumm	Platelets/Cumm	NRBC	Spleen cm	Diag.
NH	phlebotomy	54	23,000	275,000	–	10	PV
GA	myleran	30	60,000	550,000	+	absent	MMM
AB	myleran	21	18,000	20,000	+	20	PV+MMM
PO	myleran	23	8 700	700,000	+	6	MMM
SY	myleran	33	26 000	580 000	+	12	MMM
AJ	myleran	46	28 000	200,000	+	6	PV+MMM
YB	phlebotomy	56	37,000	300,000	–	N	PV
GS	phlebotomy	53	19,000	245,000	–	N	PV
FM	Myleran	29	normal	normal	+	absent	MMM
MR	phlebotomy	59	20,000	normal	–	N	PV
NS	none	55	8,300	normal	–	N	SP
ID	none	53	9 100	70,000	–	N	SP

N = Normal (not palpable) PV = polycythemia vera SP = secondary polycythemia MMM = myelofibrosis associated with myeloid metaplasia.

Table II. Erythroid colony formation by peripheral blood mononuclear cells obtained from normals (A–F) and patients with secondary polycythemia (ID NS) cells were incubated with 2 IU Epo/ml for 5–14 days[3]

Subject	Number of colonies/10^4 cells[2] Days of incubation		
	5–7	10	14
A	0	0	40 ± 5
B	0	36 ± 3	115 ± 12
C	0	12 ± 2	51 ± 4
D	0	14 ± 2	108 ± 9
E	0	4 ± 1	48 ± 5
F	0	24 ± 2	125 ± 12
ID	0	2 ± 1	75 ± 6
NS	0	29 ± 3	98 ± 11

1 Secondary polycythemia was due to chronic obstructive pulmonary disease (COPD).
2 No colony formation occurred in the absence of Epo.
3 Data represents mean ± SE for 4 separate clots.

peripheral blood mononuclear cells from all patients with myelofibrosis associated with myeloid metaplasia gave rise to large numbers of erythroid colonies in the absence of exogenous Epo Moreover endogenous ery *throid colonies appeared as early as day* 5–7 Finally the number of colonies increased in cultures containing Epo. As shown in table IV peripheral blood mononuclear cells from 4 patients with active polycythemia vera also gave rise to endogenous erythroid colonies. There was also a response to increasing concentrations of Epo between 0.25 and 2 IU/ml.

On one occasion spleen cells were obtained after splenectomy from a patient with myelofibrosis and myeloid metaplasia (FM). Examination of histological sections of this spleen revealed extramedullary hematopoiesis. A piece of spleen tissue obtained at the time of surgery was minced in HBSS,

Table III. Erythroid colony growth by peripheral blood mononuclear cells from patients with myelofibrosis

Patient	Day of incubation	Number of cells plated 10^5	Number of colonies/10^5 cells erythropoietin concentration, IU/ml			
			0	0.5	1	2
AJ	7	0.5	122 ± 13		178 ± 19	218 ± 30
SY	5	0.5	78 ± 9		100 ± 12	149 ± 17
SY	5	1.0	136 ± 15		188 ± 22	215 ± 27
SY	8	1.0	222 ± 26		267 ± 31	365 ± 42
SY	14	1.0	TNTC	TNTC		
PG	6	1.0	24 ± 3			84 ± 10
PG	10	1.0	33 ± 4			90 ± 12
AB	5	1.0	47 ± 6	82 ± 10	106 ± 13	
AB	12	1.0	TNTC	TNTC	TNTC	
FM	7	1.0	154 ± 17			236 ± 27
GA	10	1.0	88 ± 9			166 ± 19

TNTC = Too numerous to count.
Data represents mean ± SE for 4 separate clots.

Table IV. Effect of varying concentrations of erythropoietin (Epo) on erythroid colony formation by peripheral blood mononuclear cells from patients with polycythemia vera

Epo concentration, IU/ml	Number of colonies/10^5 cells Patient			
	GS	VB	NH	MR
0.00	46 ± 5	14 ± 2	65 ± 7	22 ± 4
0.25			77 ± 6	32 ± 4
0.50	149 ± 20	27 ± 3	92 ± 10	
1.00	230 ± 31	32 ± 3	138 ± 12	98 ± 10
2.00	343 ± 37	40 ± 5	193 ± 30	
4.00	260 ± 27			

Data represents mean ± SE for 4 separate clots.

strained through sterile gauze, washed, and the mononuclear cells separated on a Ficoll-Hypaque gradient. These were then plated in the usual plasma clot culture. Examinations of these cultures revealed that small numbers of erythroid colonies appeared after 5 days of growth in the absence of added Epo.

Discussion

The present study demonstrates that the peripheral blood mononuclear cell fraction obtained by density gradient centrifugation from patients with polycythemia vera and from individuals with myelofibrosis associated with myeloid metaplasia contains erythroid precursor cells of high proliferation capacity. These erythroid precursor cells form erythroid colonies which appear early in culture (5–7 days) as compared with normal peripheral blood cells (10–14 days), and also, unlike normals, appear in the absence of exogenous Epo. The ability of the peripheral blood mononuclear cells from patients with these two disorders to form er

ythroid colonies in the absence of exogenous Epo (endogenous colonies) is similar to that seen with the *in vitro* culture of polycythemia vera bone marrow [12, 16] It has been suggested that endogenous erythroid colony formation may result from a population of erythroid-committed precursors that are exquisitely sensitive to small concentrations of Epo which may be present in the fetal calf serum used in the culture system [16] Elucidation of this problem awaits the development of a synthetic medium for the cultivation of erythroid colonies In addition, unidentified myelostimulatory factors have also been suggested to be present in the serum of polycythemia vera patients [15] and the possibility that such factors could be present in our specimens is not known.

Chervenick [3] was the first to show that peripheral blood of patients with myelofibrosis associated with myeloid metaplasia contains increased numbers of cells capable of giving rise to white cell colonies (CFU_c) *in vitro* His studies also included a patient with myelofibrosis associated with myeloid metaplasia who had a previous history of polycythemia vera In addition *Ohl et al* [11] cultured peripheral blood and bone marrow cells in diffusion chambers which were implanted in the peritoneal cavities of irradiated mice and showed that peripheral blood from patients with myelofibrosis and myeloid metaplasia contains increased numbers of erythrocytic and neutrophilic progenitors. These observations are compatible with our studies on erythroid colony formation.

In contrast to the above findings, the peripheral blood mononuclear cells from patients with various secondary polycythemias failed to produce endogenous erythroid colonies These findings are consistent with the observations of *Prchal and Axelrad* [12] who were unable to demonstrate endogenous erythroid colony formation by bone marrow cells obtained from patients with secondary polycythemia. This difference in the erythroid colony-forming ability of peripheral blood mononuclear cells between polycythemia vera and secondary polycythemia may offer the potential of a useful diagnostic tool in helping distinguish between these clinical states when other diagnostic criteria are equivocal. Nevertheless, the specificity of such colony growth has to be questioned since there are rare examples of familial erythrocytosis in which spontaneous erythroid colony formation has occurred and which probably do not represent polycythemia vera [8] The present studies also demonstrate the presence of a population of cells in peripheral blood of patients with both polycythemia vera and myelofibrosis associated with myeloid metaplasia that respond to exogenous Epo in culture. Similar responsiveness to exogenous Epo has been reported for bone marrow cells obtained from patients with polycythemia vera [16] These observations are consistent with the findings of others that several lines of Epo-responsive cells exist in polycythemia vera [7]

Extramedullary hematopoiesis in patients with myelofibrosis is usually present in the spleen and is believed to be responsible for the splenic enlargement which is a major sign of the clinical syndrome of myelofibrosis with myeloid metaplasia. The role of circulating erythroid precursor cells capable of giving rise to erythroid colonies *in vitro* in the development of extramedullary hematopoiesis remains obscure. However the present study demonstrates that cells

capable of giving rise to erythroid colonies were present in the spleen of a patient with myelofibrosis associated with myeloid metaplasia. Presently we are unaware of any studies on erythroid colony growth from spleen cells obtained from normal individuals. Whether such progenitor cells are native to the spleen or arise from elsewhere remains to be determined. In this respect, data from experiments by *Goldberg et al.* [6] suggest that the spleen may be an important site of CFU production in myelofibrosis.

References

1 Adamson, J W Fialkow P J Murphy S., Prchal, J F and Steinmann, L. Polycythemia vera. the nature of the cellular origin of the disease. New Engl. J Med. *295* 913–916 1976).

2 Boyum, A. Separation of leukocytes from blood and bone marrow Scand. J clin. Lab. Invest. *21* 7–11 (1968).

3 Chervenick, P A. Increase in circulating stem cells in patients with myelofibrosis. Blood *41* 57–60 (1973).

4 Clarke, B. and Housman, D. Characterization of an erythroid precursor cell of high proliferative capacity in normal human peripheral blood. Proc. natn. Acad. Sci. USA *74* 1105–1109 (1977).

5 Glass, J L. and Wasserman, L. R. Primary polycythemia, in Williams, Beutler Erslev and Rundles, Hematology p. 527 (McGraw-Hill, New York 1972).

6 Goldberg, J Tice, D., Nelson, D Landaw S. A., and Gottlieb, A. J. The spleen as site of colony forming cell production in primary myelofibrosis. Blood *50*: 145 (1977).

7 Golde, D. W.; Bersch, N., and Cline, M. J Polycythemia vera, hormonal modulation of erythropoiesis *in vitro*. Blood *49* 399–404 (1977).

8 Greenberg, B. R. and Golde, D W.: Erythropoiesis in familial erythrocytosis. New Engl. J Med. *296*. 1080–1086 (1977).

9 Horland, A., Wolman, S., Murphy M. J and Moore, M. A. S. Proliferation of erythroid colonies in semi-solid agar Br J Haemat. *36*. 495–498 (1977).

10 Ogawa, M.; Grush, O. C.; O'Dell, R. F Hara, H., and MacEachern, M. D.: Circulating erythropoietic precursors assessed in culture: characterization in normal men and patients with hemoglobinopathies. Blood *50* 1081 1086 (1977).

11 Ohl, S.; Carsten, A. L., Chanana, A. D; Chikkappa, G., and Cronkite, E. P Increased erythrocytic and neutrophilic progenitors in myelofibrosis with myeloid metaplasia. Eur J Cancer *12*: 131–136 (1976).

12 Prchal, J F and Axelrad, A. A. Bone marrow response in polycythemia vera. New Engl. J Med. 290 1382 1383 (1974).

13 Prchal, J F.; Axelrad, A. A., and Crookston, J H.: Erythroid colony formation in plasma culture from cells of peripheral blood in myeloproliferative disorders. Blood *44* 912 (1974).

14 Stephenson, J R.; Axelrad, A. A.; McLeod, D. L., and Shreeve, M. M. Induction of colonies of hemoglobin-synthesizing cells by erythropoietin *in vitro* Proc. natn. Acad. Sci. USA *68*. 1542–1545 (1971).

15 Zanjani, E. D. Hematopoietic factors in polycythemia vera. Semin. Hematol. *13*. 1–12 (1976).

16 Zanjani, E. D Lutton, J D., Hoffman, R., and Wasserman, L. R. Erythroid colony formation by polycythemia vera bone marrow *in vitro*. J clin. Invest. *59*· 841–846 (1977).

Accepted: March 20, 1979

J D Lutton, PhD, Department of Medicine,
New York Medical College,
Valhalla, NY 10595 (USA)

Acta haemat. 62 100–105 (1979)

Serum Levels of Retinol, Retinol-Binding Protein, Carotenoids and Triglycerides in Children with β-Thalassemia major

Ch. Katerelos A Constantopoulos A Agathopoulos N Constantzas Lida Zannos Mariolea and N Matsaniotis

1st Department of Pediatrics, University of Athens and Hagia Sophia Children's Hospital, Athens

Key Words. Carotenoids Retinol Retinol Binding Protein Thalassemia Triglycerides

Abstract. Levels of retinol (vitamin A) carotenoids and triglycerides in the serum of 50 children with homozygous β-thalassemia have been studied, as well as the ability of the small intestine to absorb a test meal containing retinol palmitate, triglyceride, *d* xylose and glucose. On the other hand, 8 patients underwent a dark-adaptation test, and in 40 children with homozygous β-thalassemia the levels of retinol-binding protein in the serum were estimated. The mean levels of *retinol, carotenoids and triglycerides* in the serum of the patients were 23 ± 4 1 μg/dl (controls 36.3 ± 4.9) 44 ± 15.5 μg/dl (controls 103 ± 24) 117 ± 20 (controls. 126 ± 26), respectively The absorption from the small *intestine of retinol triglycerides, glucose and d-xylose was normal. 6 out of 8 patients* studied for visual function showed an abnormal dark-adaptation test, and these 6 children had low serum retinol levels. Finally the mean serum levels of retinol-binding protein in *the patients were 4 74 ± 0.53 mg/dl (controls 5 63 ± 0.58) The low retinol levels* were correlated with the low retinol-binding protein values which, in turn, could be due to the abnormal liver function of the patients.

Previous studies in this laboratory and *elsewhere have shown decreased serum to-*copherol levels and increased susceptibility of red cells to auto-oxidation in patients *homozygous for β* thalassemia [11 14] In testinal malabsorption of vitamin E was one among many other hypotheses advanced to *explain* the above findings. Studies on intestinal absorption in Greek children with homozygous β-thalassemia, carried out in other laboratories have shown contradictory results [4 10] For this reason further research on the intestinal function in this disease *was deemed necessary The present in*vestigation comprises two parts. The first part includes measurements of serum retinol, carotenoids and triglycerides and the evaluation of the ability of the small intestine to absorb a test meal containing retinol palmitate, triglyceride, *d* xylose and glucose in 50 children with homozygous β-thalassemia. The second part consists in dark adaptation tests and measurements of retinol-binding protein.

Material and Methods

Control Group

50 children aged 3–12 years (mean 6.9 ± 2.1) were used as controls. They were selected among patients referred to the Orthopedic Clinic for variety of problems none of which affected their nutritional status. Their weights ranged between the 50th and 75th percentile. Hemoglobin values ranged between 10.0 and 13.0 g/dl (mean 11.97 ± 0.64). None of them was heterozygous for β-thalassemia.

Patients

There were 50 children aged 3–12 years (mean 6.5 ± 2.2) suffering from homozygous β-thalassemia. Their weights ranged between the 25th and 50th percentile. Hemoglobin values ranged between 5.2 and 8.1 g/dl (mean 6.44 ± 0.70). The children originated from Greek provinces located far from Athens and this was the main reason why they were not transfused more frequently as indicated in order to keep their hemoglobin levels above 8.5 g/dl. The last transfusion was performed 30–40 days before the evaluation. The diagnosis of homozygous β-thalassemia was made by established hematological criteria.

Children of both groups were admitted to the hospital for 4 h. All of them were reported to have good appetite and normal daily nutritional intake. Neither the quantity nor the quality of fat, carbohydrate or protein consumed differed significantly in the two groups. Blood was drawn after 12 h fasting.

Assay of Retinol and Carotenoids

Serum concentration of retinol and carotenoids was determined by the micromethods of *Bessey et al.* [2] and *Kaser and Stekel* [7], respectively. Briefly 1 ml serum was diluted with 2 ml absolute ethanol and extracted with 2 ml n-heptane. The top layer of n-heptane was divided into two tubes. One tube was put in dark place, and the other was irradiated with ultraviolet light for 3 h. Finally reading was done at 327 and 452 nm. The values of retinol and carotenoids were estimated with standard curve.

Determination of Serum Triglycerides

The kit of Boehringer Mannheim, was used for the determination of triglycerides.

Assay of Intestinal Absorption

A test meal was administered, containing 2 g of glucose per kilogram body weight (not less than 20 g), 0.5 g/kg of d-xylose and 2,250 μg/kg (7,500 IU) of retinol palmitate. No drinks or food were allowed for the next 5 h.

Blood was drawn before the test meal and 30, 60, 90, 120, 240, 360 and 480 min thereafter and analyzed for glucose, d-xylose, triglycerides and retinol.

Dark-Adaptation Test

Dark-adaptation studies were performed on Goldmann-Weekers adaptometer. Each subject was initially adapted to the dark room for 20 min, after which threshold measurements were started immediately. Dark adaptation was usually completed within 25 min.

An ascending threshold was the intensity at which the subject first saw the test light as its luminance was increased; descending threshold was the intensity at which the subject ceased to see the test light as its luminance was lowered. The logarithm of the light-perception threshold is plotted as a function of time in darkness, the change in threshold follows a characteristic course. There is an initial rapid fall in threshold attributed to the cones, followed by the cone plateau. With the method used in this study the rod-cone break occurs at 3–6 min, followed by a slower descent attributed to the rods. The final threshold attained in about 25 min is the most constant indicator of function and is the measure of retinol function used here [3].

Determination of Retinol-Binding Protein in Serum

The serum levels of retinol-binding protein were measured by simple radial immunodiffusion using commercial plates (M-Partigen, Behringwerke), with diffusion time of 24 h at room temperature. Free and bound retinol-binding protein was measured by this method.

Results

Serum Retinol, Carotenoid and Triglyceride Levels

50 controls and 50 patients were examined. Serum retinol, carotenoid and triglyceride values are shown in figure 1

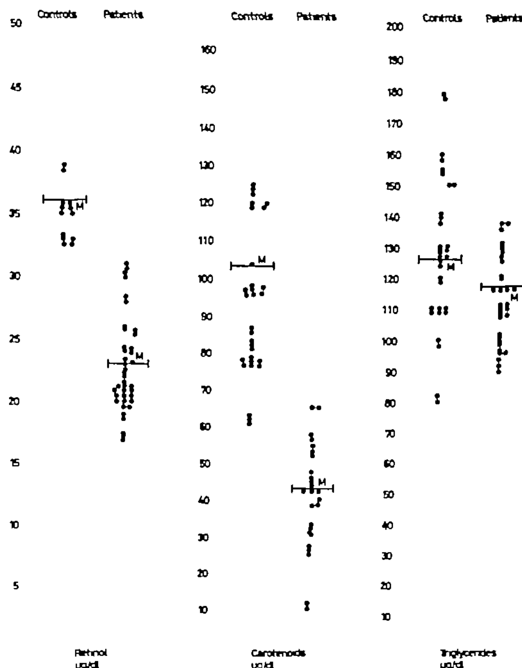


Fig. 1. Serum levels of retinol, carotenoids and triglycerides, in 50 patients and 50 controls. The mean (M) levels of retinol, carotenoids and triglycerides in the serum of the patients were 23 ± 4.1 μg/dl (controls: 36.3 ± 4.9), 44 ± 15.5 μg/dl (controls: 103 ± 24) 117 ± 20 mg/dl (controls: 126 ± 26), respectively. The difference between the two groups is highly significant for retinol and carotenoids ($p < 0.001$).

Intestinal Absorption (Test Meal)

The test meal was given to 7 children from each group chosen at random. Serum retinol levels before the test meal were 39.1 ± 3.4 μg/dl in the controls and 24.5 ± 6.1 μg/dl in the patients (fig. 2).

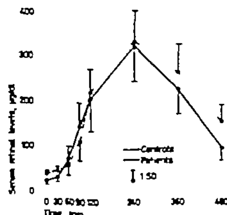


Fig. 2. Serum retinol levels before and after administration of test meal. The mean values of retinol before the test meal were 24.5 ± 6.1 μg/dl (controls: 39.1 ± 3.4). The difference is highly significant ($p < 0.001$). After administration of a test meal the retinol levels were almost similar in the two groups, although they dropped faster in the patients.

Levels reached a peak 240 min after the administration of the test meal and were similar in both groups (fig. 2). Retinol levels dropped faster in the patients as compared to controls.

Serum triglyceride, *d* xylose and glucose levels before and after the test meal were *similar* in both groups (*data not shown*).

Dark Adaptation Capacity

8 patients with β-thalassemia major underwent dark adaptation testing. 6 out of these 8 patients showed abnormally high final thresholds ranging from 4 10[4] to 3.10[6] psb.

The correlation of dark adaptation and retinol in the serum in these 8 patients is shown in figure 3

Levels of Retinol-Binding Protein

The levels of retinol-binding protein for patients and controls are shown in figure 4

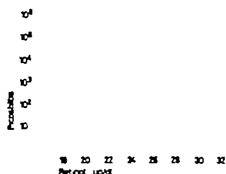


Fig. 3. Correlation of dark-adaptation and retinol in the serum of 8 patients. 6 patients with abnormal dark-adaptation have low levels of serum vitamin A. The other 2 patients who have normal dark-adaptation, also have normal retinol levels.



Fig. 4. Levels of retinol-binding protein in 40 patients and 40 controls. The mean (M) levels of retinol-binding protein were 4.74 ± 0.53 and 5.63 ± 0.58 mg/dl for patients and controls, respectively. This difference is statistically highly significant ($p < 0.001$).

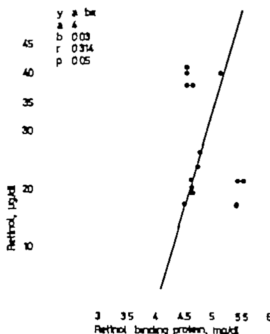


Fig. 5. Correlation of retinol and retinol-binding protein in 40 patients.

Correlation of Retinol and Retinol Binding Protein

There was a very good correlation between retinol and retinol-binding protein (fig. 5)

Discussion

This is the first study of retinol serum levels in normal Greek children. Values ranged within the limits reported as normal in the literature, but the mean level was somewhat below that found in well nourished American children reflecting probably the lower consumption of milk and butter by Greek children in general [6 8] Retinol levels were found to be significantly lower than normal in the children with homozygous β-thalassemia. The possibility of a low vitamin A intake seems improbable in view of the fact that the diet of the thalassemic patients did not differ from that of the normal children and that their appetite was good as reported by the parents. Intestinal malabsorption was ruled out with the finding of normal absorption of retinol, triglycerides, *d* xylose and glucose. Defective mobilization of retinol from the liver could account for the low serum values. It is well established that retinol is transported out of the liver to the plasma and is associated with a transfer protein called retinol-binding protein [5] Deficiency in this protein results in low retinol serum levels in spite of normal liver vitamin A. Decreased synthesis of retinol-binding protein occurs when dietary protein is not adequate and in liver disease [1 12] Unless an adequate pool is available in the liver retinol binding protein cannot be synthesized [1 12] Serum proteins were not evaluated in our children but, as already mentioned, their protein intake did not appear to differ from that of the control group.

On the other hand, liver function is abnormal in thalassemic patients [13] Theoretically increased excretion or overutilization of retinol could also explain the low serum levels of this vitamin.

The low serum carotenoid levels of our patients with thalassemia are difficult to explain. The usual factors affecting carotenoid levels, namely low intake of carotenoids and fat or fat malabsorption were ruled out. Other explanations would be low β-lipoprotein levels or anemia. Low β-lipoprotein levels have been found in adults with heterozygous β-thalassemia but not in children [4 10] To our knowledge, β-lipoprotein levels in children with homozygous β-thalassemia have not been reported.

Naiman et al [9] found low serum carotenoid levels in children suffering from anemia of various etiologies. Among them were

3 patients homozygous for β-thalassemia. Correction of the anemia in the children with iron deficiency who also had extensive abnormalities of the gastrointestinal structure and function, resulted in normal serum carotenoid levels, although the vitamin A tolerance curve remained flat. These findings would be compatible with the hypothesis that, for some unknown reason, anemia *per se* might be responsible for low carotenoid levels.

In the second part of this study we evaluated the vitamin A status of the patients with homozygous β-thalassemia. None of the 8 patients complained of night blindness, although an initial functional deficit was demonstrated by dark adaptation testing in 6 patients. Other vitamin deficiencies have also been reported to produce delayed dark adaptation (riboflavine and vitamin C) [3] therefore, it remains to be seen whether this abnormality will be reversed with the administration of vitamin A.

The low retinol-binding protein levels observed in the patients with thalassemia could be due either to accumulation of this protein in a vitamin-A-deficient liver or to defective synthesis, as mentioned previously. In any case, the finding of low retinol binding protein does not answer the question whether the low serum retinol levels in homozygous β-thalassemia represent a deficiency in the vitamin or not.

References

1 Arroyave, G., Wilson, D. Contreras, C., and Behar, M.: Alterations in human concentration of vitamin A associated with hypoproteinemia in severe protein malnutrition. J. Pediat. 62: 920–925 (1963).

2 Bessey, O. A.; Lowry, O. H., Brock, M. J. and Lopez, J. A.: Determination of vitamin A and carotene in small quantities of blood serum. J. biol. Chem. *116*: 177–181 (1946).

3 Duke-Elder, S. E.: System of opthalmology vol. 7, pp. 384–386 (Kimpton, London 1962).

4 Fessas, P. Stamatoyannopoulos, G. and Keys, A.: Serum cholesterol and thalassemia trait. Lancet *i*: 1182–1183 (1963).

5 Ganguly, J. Absorption, transport and storage of vitamin A. Vitams Horm. *18*: 387–403 (1960).

6 Hodges, R. E. and Krehl, W. A.: Nutritional study of teenagers in Iowa. Am. J. clin. Nutr. *17*: 200–204 (1965).

7 Kaser, M. and Stekol, J. A.: J. Lab. clin. Med. *28*: 904 (1943); cited by Varley: practical clinical biochemistry; 3rd ed., pp. 508–532 (Heinemann, London 1963).

8 Morse, E. H., Merrow, S. B., and Clarke, R. E.: Some biochemical findings in Burlington Junior High School children. Am. J. clin. Nutr. *17*: 211–218 (1965).

9 Naiman, J. L., Oski, F. A., Diamond, L. K., Vawter, G. F. and Schwachman, H.: The gastrointestinal effects of iron-deficiency anemia. Pediatrics, Springfield *33*: 83–99 (1964).

10 Panteiakis, S. and Doxiadis, S. A.: Serum lipoproteins in schoolboys in relation to glucose-6-phosphate dehydrogenase deficiency and thalassemia trait. Archs Dis. Child. *42*: 326–331 (1967).

11 Stocks, J. Offerman, E. L., Modell, C. B., and Dormandy, T. L.: The susceptibility to autoxidation of human red cell lipids in health and disease. Br. J. Haemat. *23*: 713–720 (1972).

12 Underwood, B. A. and Denning, C. R.: Blood and liver concentrations of vitamins A and E in children with cystic fibrosis of the pancreas. Pediat. Res. *6*: 26–31 (1972).

13 Weatherall, D. J. and Clegg, J. B.: The thalassemia syndromes; 2nd ed., p. 91 (Blackwell, Oxford 1972).

14 Zannos-Mariolea, L., Tzortzatou, F.; Dendaki-Svolaki, K., Katerelos, C., Kavallari, M., and Matsaniotis, N. Serum vitamin E levels in children with β-thalassemia. Br. J. Haemat. *26*: 193–199 (1974).

Received: August 29, 1978
Accepted: February 10, 1979

Dr. A. Constantopoulos, Aghia Sophia Children's Hospital, Athens 608 (Greece)

Acta haemat. 62. 106-111 (1979)

Interaction between β-Thalassaemia and Hb G Philadelphia Associated with α-Thalassaemia

Regina Stathopoulou E Sulis and H Lehmann

University Department of Biochemistry Cambridge, and Ospedale Oncologico, Cagliari

Key Words. Globin biosynthesis Hb G Philadelphia α-Thalassaemia β-Thalassaemia

Abstract. A man who did not produce any β-chains did not suffer from a severe β-thalassaemia. He was heterozygous for Hb G Philadelphia. It has been suggested that this haemoglobin variant was associated with α-thalassaemia and that interaction between α-thalassaemia and β-thalassaemia decreased the imbalance of α/β-globin biosynthesis and thereby the severity of the β-thalassaemic disorder Association of Hb G Philadelphia and α-thalassaemia in this man and his family is now demonstrated using bone marrow and reticulocytes of the propositus and one of his sons and reticulocytes only of another son.

In 1975 a family was described where the propositus seemingly had β-thalassaemia major [1] yet the clinical course was milder than that expected for this condition The propositus was 59 years old and though there was an anaemia of 10 g haemoglobin per decilitre with spleno- and hepatomegaly and a history of leg ulcers, he had been fit to work all his life and had never required a blood transfusion. There were two types of α-chains present, and in the absence of β-chains the haemoglobins found were Hbs F and A_2 with the α-chains being either α^A or α^G (G Philadelphia) At that time attention had been drawn to an association between *Hb G Philadelphia and α-thalassaemia* [2] and it was known that the severity of β-chain deficiency might be modified by a concurrent α-thalassaemia [3] An α-thalassaemia would reduce the surplus of free α-chains associated with β-thalassaemia, and because the degree of haemolysis is related to the amount of free α-subunits, their reduction would make the β-thalassaemia less severe Whether however the relatively good clinical state in this apparent case of β^0 thalassaemia major could be related to an α-thalassaemia associated with the Hb G Philadelphia had to wait for a study of the α/non-α-globin chain synthesis in this man and in members of his family

In this report we describe globin biosynthetic studies in the propositus and two of his sons.

Methods

Haematological evaluations were made by standard techniques [4], and haemoglobin electrophoresis was carried out on paper at pH 8.9 [5]. The various haemoglobin components were quantitated by DEAE-cellulose chromatography [6] and by elution following Kohn cellulose acetate electrophoresis of haemolysates [7]. Quantitation of Hb F below 20% was performed by alkali denaturation [8], and above 20% by DEAE-cellulose chromatography. The intracellular distribution of Hb F was examined by acid elution [9]. Structural studies of whole globin and of separated globin chains [10] followed methods summarized previously [11].

The relative rates of α/non-α-globin synthesis were measured *in vitro* by the incorporation of [³H] leucine into bone marrow cells and into reticulocyte-enriched preparations of red cells according to the method of *Lingrel and Borsook* [12] with some minor modifications [13].

The α-chain excess was quantitated using gel filtration on Sephadex G-100 with Tris/HCl buffer (pH 7.4) at 4 °C [14].

Results

The propositus and two of his sons were reinvestigated in the present study. For completeness, the haematological findings and data on the haemoglobin composition of the whole family as described before [1] are included in table I.

Propositus

The distribution of Hb F in the red cells was homogeneous in the propositus and the ratio of $^{G}\gamma$:$^{A}\gamma$ chains was 2:1:1. As previously stated, an incidental finding was a previously not described mutation in his fetal haemoglobin: γ75 Ile-Thr (Sardinia) [1]. It had not been possible to determine chemically whether this mutation was present in the $^{A}\gamma$-chain, the $^{G}\gamma$-chain or in both. There were two α-chains present, [illegible] and α^{G} Philadelphia (α68 Asn → Lys) and the proportion of α^{G} was 34% of the total α-chains. There were present two fetal haemoglobins $\alpha^{A}{}_{2}\gamma$ and $\alpha^{G}{}_{2}\gamma_{2}$, and two Hbs A_2 were found [illegible]$_{2}\delta_{2}$ and $\alpha^{G}{}_{2}\delta_{2}$. The two Hbs A_2 amounted to 1% of the total haemoglobin.

Family

Two sons of the propositus (III 1 and III-2) were reinvestigated. Like the propositus' wife, his father, his third son and his daughter they too were in good health with no abnormal clinical findings.

They were heterozygotes for Hb G Philadelphia and the proportion of this haemoglobin in the red cells was found by ion-exchange chromatography to be 35 and 43% of the total haemoglobin, respectively. In both the level of Hb F was above the normal range (table I).

In vitro *Biosynthesis*

Labelled reticulocytes as well as labelled bone marrow cells from the propositus and one son (III 2), and reticulocytes only from the other son (*III 1*) were used.

Figure 1 shows the incorporation of [³H] leucine into the various globin chains during

Table I. Haematological values and composition of haemoglobin

	RBC 10^{12}/l	Hb g/dl	PCV l/l	MCV fl	MCH pg	MCHC g/dl	Hb F %	Hb A_2 %	Hb G Phil. %
Propositus (II 1)	4.1	10.2	0.30	76	26	33	79	1	34.0
Father (I-1)	5.1	15.4	0.50	98	30	31	4	8	present
Wife (II-2)	5.6	14.6	0.49	86	26	30			
Son (III 1)	5.1	15.4	0.50	98	30	31	4.3	9.2	35.0
Son (III 2)	5.6	14.3	0.44	80	26	33	7.0	9.8	43.0
Son (III-3)	4.8	15.7	0.46	96	33	34	–	–	
Daughter (III-4)	3.7	13.2	–				6.3	7.8	

Acta haemat. 62: 106–111 (1979)

Interaction between β-Thalassaemia and Hb G Philadelphia Associated with α-Thalassaemia

Regina Stathopoulou, E. Sulis and H. Lehmann

University Department of Biochemistry, Cambridge, and Ospedale Oncologico, Cagliari

Key Words. Globin biosynthesis · Hb G Philadelphia · α-Thalassaemia · β-Thalassaemia

Abstract. A man who did not produce any β-chains did not suffer from a severe β-thalassaemia. He was heterozygous for Hb G Philadelphia. It has been suggested that this haemoglobin variant was associated with α-thalassaemia and that interaction between α-thalassaemia and β-thalassaemia decreased the imbalance of α/β-globin biosynthesis and thereby the severity of the β-thalassaemic disorder. Association of Hb G Philadelphia and α-thalassaemia in this man and his family is now demonstrated using bone marrow and reticulocytes of the propositus and one of his sons and reticulocytes only of another son.

In 1975 a family was described where the propositus seemingly had β-thalassaemia major [1], yet the clinical course was milder than that expected for this condition. The propositus was 59 years old and though there was an anaemia of 10 g haemoglobin per decilitre with spleno- and hepatomegaly and a history of leg ulcers, he had been fit to work all his life and had never required a blood transfusion. There were two types of α-chains present, and in the absence of β-chains the haemoglobins found were Hbs F and A_2 with the α-chains being either α^A or α^G (G Philadelphia). At that time, attention had been drawn to an association between Hb G Philadelphia and α-thalassaemia [2] and it was known that the severity of β-chain deficiency might be modified by a concurrent α-thalassaemia [3]. An α-thalassaemia would reduce the surplus of free α-chains associated with β-thalassaemia, and because the degree of haemolysis is related to the amount of free α-subunits, their reduction would make the β-thalassaemia less severe. Whether, however, the relatively good clinical state in this apparent case of β^0-thalassaemia major could be related to an α-thalassaemia associated with the Hb G Philadelphia had to wait for a study of the α/non-α-globin chain synthesis in this man and in members of his family.

In this report we describe globin biosynthetic studies in the propositus and two of his sons.

Table II. Globin biosynthesis ratios

Subject and sample	Total count ratios, cpm				Specific activity ratios, cpm/OD			
	$\frac{\alpha^A+\alpha^G}{\text{non-}\alpha}$	$\frac{\alpha^A+\alpha^G}{\beta+\gamma}$	$\frac{\alpha^A+\alpha^G}{\beta}$	$\frac{\alpha^G}{\alpha^A+\alpha^G}$	α^A/γ	α^G/γ	α^A/β	α^G/β
Propositus (II-1)								
Reticulocytes	1.38		–	0.34	1.83	1.86		
Bone marrow	1.08	–		0.30	1.34	0.96	–	
Son (III-1)								
Reticulocytes		0.70	1.16	0.35	–	–	1.11	0.79
Son (III-2)								
Reticulocytes		0.89	1.22	0.43		–	1.12	1.15
Bone marrow		1.31		0.42				

lates to that part of the bone marrow globin which eluted as a tetramer on gel filtration and was then separated into the different globin chains. The globin dimers and monomers consisted mainly of α-chains with a small proportion of γ-chains in the dimer. On correction for the radioactivity in the γ-chains the total free α-chain radioactivity amounted to 83% in the bone marrow cells and 54% in reticulocytes, respectively (table II).

In the propositus and his son the synthesis of each chain as estimated from the total counts per minute (cpm) showed a balanced synthesis of α- and non-α-chains in the bone marrow samples and a moderately imbalanced α/β-globin synthesis in the reticulocytes. The globin synthesis ratio of α^A to α^G-chains was 2.1 in the propositus bone marrow and reticulocytes, which corresponds to the proportion of α^A/α^G in the red cells (table I).

δ-Chains normally elute after the γ-chain. In the reticulocytes there was no detectable δ-chain radioactivity (fig. 1). This was expected since the synthesis of this chain is confined to the bone marrow [15]. In the bone marrow (fig. 2), however, the radioactivity incorporated in the region of the δ-chain peak was estimated to be 24.7% of the total radioactivity incorporated in the non-α-chains. Attempts to confirm the chemical composition of the peak as a δ-chain by fingerprinting were unsuccessful because the amount of protein under this peak was too small for reliable analysis. It was carried out, however, in the δ-chains of the haemolysate of the two sons of the propositus. They were found to be normal δ-chains.

In the reticulocytes of the two sons a moderate imbalance of α/non-α-chain synthesis was found (table II). The α/β-globin chain synthesis ratios of 1.16 and 1.22 were higher than our non-thalassaemic control value of 1.05 ± 0.05 but well below our values of β-thalassaemia trait of 1.50–2.0. When the γ-chain counts in addition to those of the β-chain were taken into consideration, the ratios were 0.70 (III 1) and 0.89 (III 2). The first is what we usually find in α-thalassaemia type 1 and the second is on the borderline of thalassaemia type 1 and normality.

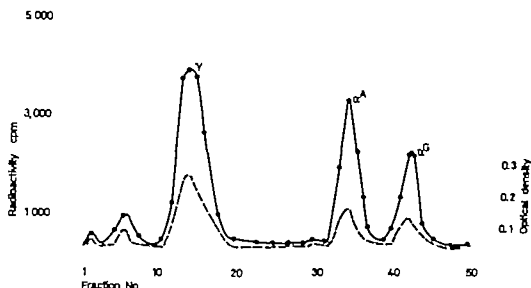


Fig. 1. Radioactivity and optical density of separated globin chains after incubation of the propositus' reticulocyte lysate with [³H] leucine ——— = Radioactivity· - - - = optical density

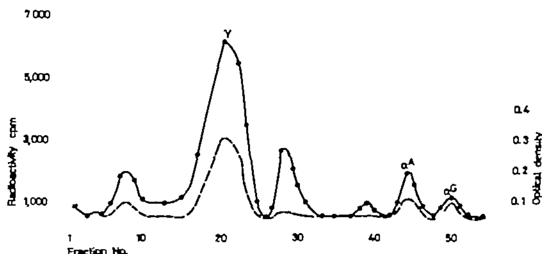


Fig. 2. Incubation of the propositus' bone marrow with [³H] leucine. The tetramers were eluted by gel filtration and their separation into the constituent globin chains is shown. ——— = Radioactivity· - - - = optical density

the incubation of the propositus reticulocytes No β-chains are synthesised and the ratio of incorporation of radioactivity into α-non-α-globin is 1.38 Further details are given in table II, which also records the results of the incubation of labelled reticulocytes from both sons.

The bone marrow of the propositus showed an α/non-α-globin synthesis ratio of 1 08 This ratio is more nearly normal than that found in the reticulocytes. Part of this difference is due to an additional non-α-globin peak in the position of the δ-chains. This peak can be seen in figure 2 which re-

relatives, but it is of course not possible to exclude that a non-haemoglobin protein was produced by the propositus bone marrow which eluted in the position usually occupied by the δ-chain.

References

1 Grifoni, V Kamuzora, H., Lehmann, H., and Charlesworth, D A new Hb variant: Hb F Sardinia $\gamma^{75(E19)}$ isoleucine-threonine found in family with Hb G Philadelphia, β-chain deficiency and a Lepore-like haemoglobin indistinguishable from Hb A_2. Acta haemat. 53 347–355 (1975)

2 French, E. A. and Lehmann, H Is haemoglobin G_{α} Philadelphia linked to α-thalassaemia? Acta haemat. 46. 149–156 (1971)

3 Knox Macaulay H. H. M., Weatherall, D. J Clegg, J B Bradley J and Brown, M. J The clinical and biosynthetic characterization of αβ-thalassaemia. Br J Haemat. 22. 497–512 (1972)

4 Dacie, J V and Lewis, S. M. Practical haematology 5th ed. (Churchill, London 1975)

5 Gradock Watson, J E., Fenton, J C. B and Lehmann, H Tris buffer for the demonstration of haemoglobin A_2 by paper electrophoresis. J clin. Path 12 372 373 (1979).

6 Huisman, T H. J and Dozy A. M Studies on the heterogeneity of hemoglobin IX. The use of Tris (hydroxymethyl) amino methane-HCl buffer in the anion exchange-chromatography of hemoglobins J Chromat. 19 160–169 (1965).

7 Marengo-Rowe, A. J Rapid electrophoresis and quantitation of haemoglobins in cellulose acetate. J clin. Path. 18. 790–792 (1965).

8 Betke, K., Marti, H. R and Schlicht, L. Estimation of small percentages of fetal haemoglobin. Nature, Lond. 184 1877–1878 (1959).

9 Kleihauer E., Braun, H and Betke, K. Demonstration von fetalem Hämoglobin in den Erythrocyten eines Blutausstrichs. Klin. Wschr 35 637–638 (1957).

10 Clegg, J B., Naughton, M. A., and Weatherall, D J Abnormal human haemoglobins. Separation and characterisation of the α- and β-chains by chromatography and the determination of two new variants. Hb Chesapeake and Hb J (Bangkok). J molec. Biol. 19 91 108 (1966).

11 Sick, K.; Beale, D Irvine, D Lehmann, H., Goodall, P T and McDougall, S.: Haemoglobin G Copenhagen and haemoglobin J Cambridge. Two new variants of haemoglobin A. Biochim. biophys. Acta 140 231 242 (1967)

12 Lingrel, J B. and Borsook, H. A comparison of amino acid incorporation into the hemoglobin and ribosome of marrow erythroid cells and circulating reticulocytes of severely anemic rabbits. Biochemistry N Y 2 309–314 (1963).

13 El Hazmi, M. A. F and Lehmann, H. Hemoglobin Riyadh α_2 β (120 (GH3) Lys → Asn). A new variant found in association with α-thalassemia and iron deficiency Hemoglobin 1 59–74 (1976/1977).

14 Modell, C. B., Latter A., Steadman, J H., and Huehns, E. R. Haemoglobin synthesis in β-thalassaemia. Br J Haemat. 17 485–501 (1969).

15 Roberts, A. V Weatherall, D J and Clegg, J B. The synthesis of human hemoglobin A_2 during erythroid maturation. Biochem. biophys. Res. Commun. 47 81–87 (1972).

16 McCurdy P R., Sherman, A. S., Kamuzora, H., and Lehmann, H.: Globin synthesis in subjects doubly heterozygous for hemoglobin G Philadelphia and hemoglobins S or C. J Lab. clin. Med. 85 891–897 (1975).

17 Politis-Tsegos, C.; Lang, A., Stathopoulou, R., and Lehmann, H.: Is haemoglobin G_{α} Philadelphia linked to α-thalassaemia? Further evidence. Humangenetik 31 67–74 (1976).

18 Kan, Y W and Nathan, G Mild thalassemia: the result of interactions of alpha- and beta-thalassemia genes. J clin. Invest. 49 635–642 (1970).

19 Lehmann, H. and Lang, A. Haemoglobin Q and thalassaemia. J sci. Soc. Thailand 1 41–48 (1975).

Received: January 3, 1979
Accepted, February 15, 1979

Prof. H. Lehmann, Department of Biochemistry
Tennis Court Road, Cambridge
CB2 1QW (England)

The ratio of α^A to α^Q radioactivity was 2 1 in one son (III 1) somewhat lower in the other (III 2) From these data it appears that both siblings have inherited from the propositus the genes for α-thalassaemia and α^Q Philadelphia.

Discussion

The combined haematological and genetic information indicates that the propositus is a heterozygote for α^Q Philadelphia plus α-thalassaemia type 2, i.e one gene for α-thalassaemia, and either two for β^o thalassaemia, or one for β^o and one for δβ-thalassaemia.

The presence of α-thalassaemia is supported by the fact that the proportion of α^Q-chains is a third of the total α-chains. It has been pointed out by *French and Lehmann* [2] and later by *McCurdy et al* [16] and *Politis Tsegos et al* [17] that the proportion of more than 25% of this Hb in heterozygotes can be explained by its association with α-thalassaemia.

The mild degree of chain imbalance (α/non-α 1.38) in the propositus as compared with the β^o-thalassaemia or even β^+ thalassaemia trait, in which the recorded α-non-α ratios are higher than 1 70 almost certainly results from the co-inherited α-thalassaemia. The co-inheritance of both α and β-thalassaemia and its clinical and haematological features have been described before [3 18, 19]

The nature of the β-thalassaemia in this case, however is not clear The absence of β-chains at first suggests that it is of β^o-type. No data are available for the propositus mother but his father and two of his sons are carriers of a β-thalassaemia-like trait since their Hb F and Hb A_2 levels are high.

For the propositus, the differential diagnosis must be made between a homozygous β^o thalassaemia and for heterozygosity for a gene responsible for β^o-thalassaemia and one for δβ-thalassaemia or a Lepore-like condition. The low Hb A_2 level in the propositus is more compatible with δβ-thalassaemia but the Hb A_2 level in his father and his sons is unusually high and suggests that it represents a Lepore-like haemoglobin. The chemical analysis shows that the Hb A_2 consists of normal δ-chains. However this does not exclude a Lepore chain, if the δβ-crossing-over occurred after residue 126 This would result in what is chemically a δ-chain, but genetically a δ/β- or Lepore chain. This could possibly explain the high radioactivity incorporated in the region where δ-chains elute in the propositus bone marrow sample which amounted to 25% of total non-α-radioactivity (fig. 2). This is a high value and δ-chain incorporation values obtained from thalassaemic bone marrow cells in our laboratory averaged no more than 10% of the total non-α-chain values. It is unlikely that this peak is due to an abnormal β-chain since it was not detected in reticulocytes and mature red cells. Thus the Hb A_2 chain could be chemically a δ-chain but genetically a δβ-chain, with a relatively small amount of structural β-mRNA only being responsible for the C terminus. The difference between the levels of the δ-chain (low in the propositus and high in his first-degree relatives) would then have its explanation in the absence of interaction with any β-chain production in the propositus. These considerations are based on the very high Hb A_2 values found in the propositus first-degree

relatives, but it is of course not possible to exclude that a non-haemoglobin protein was produced by the propositus bone marrow which eluted in the position usually occupied by the δ-chain.

References

1 Grifoni, V Kamuzora, H., Lehmann, H., and Charlesworth, D A new Hb variant: Hb F Sardinia $\gamma^{75(E1)}$ isoleucine-threonine found in family with Hb G Philadelphia, β-chain deficiency and Lepore-like haemoglobin indistinguishable from Hb A_2. Acta haemat. *53* 347–355 (1975).

2 French, E. A. and Lehmann, H Is haemoglobin G_α Philadelphia linked to α-thalassaemia? Acta haemat. *46* 149–156 (1971)

3 Knox Macaulay H. H. M., Weatherall, D. J Clegg, J B., Bradley J and Brown, M. J The clinical and biosynthetic characterisation of αβ-thalassaemia. Br J Haemat. *22*. 497–512 (1972).

4 Dacie, J V and Lewis, S. M. Practical haematology 5th ed. (Churchill, London 1975).

5 Gradock Watson, J E., Fenton, J C B., and Lehmann, H. Tris buffer for the demonstration of haemoglobin A by paper electrophoresis. J clin. Path. *12* 372 373 (1979).

6 Huisman, T H. J and Dozy A. M Studies on the heterogeneity of hemoglobin IX. The use of Tris (hydroxymethyl) amino methane-HCl buffer in the anion exchange-chromatography of hemoglobins. J Chromat. *19*· 160–169 (1965)

7 Marengo-Rowe, A. J Rapid electrophoresis and quantitation of haemoglobins in cellulose acetate. J clin. Path. *18*. 790–792 (1965).

8 Betke, K., Marti, H R., and Schlicht, L. Estimation of small percentages of fetal haemoglobin. Nature, Lond. *184* 1877–1878 (1959).

9 Kleihauer E., Braun, H and Betke, K.. Demonstration von fetalem Hämoglobin in den Erythrocyten eines Blutausstriche. Klin. Wschr *35*. 637–638 (1957).

10 Clegg, J B.; Naughton, M. A., and Weatherall, D J Abnormal human haemoglobins. Separation and characterisation of the α- and β-chains by chromatography and the determination of two new variants. Hb Chesapeake and Hb J (Bangkok). J molec. Biol. *19* 91 108 (1966).

11 Sick, K. Beale, D., Irvine, D Lehmann, H., Goodall, P T and McDougall, S. Haemoglobin G Copenhagen and haemoglobin J Cambridge. Two new variants of haemoglobin A. Biochim. biophys. Acta *140*· 231 242 (1967).

12 Lingrel, J B. and Borsook, H.. A comparison of amino acid incorporation into the hemoglobin and ribosome of marrow erythroid cells and circulating reticulocytes of severely anemic rabbits. Biochemistry N Y *2*. 309–314 (1963).

13 El Hazmi, M. A. F and Lehmann, H.. Hemoglobin Riyadh α_2 β_2 (120 (GH3) Lys → Asn). A new variant found in association with α-thalassemia and iron deficiency Hemoglobin *1* 59–74 (1976/1977).

14 Modell, C. B., Latter, A., Steadman, J H., and Huehns, E. R.. Haemoglobin synthesis in β-thalassaemia. Br J Haemat. *17* 485–501 (1969).

15 Roberts, A. V Weatherall, D J and Clegg, J B.. The synthesis of human hemoglobin A_2 during erythroid maturation. Biochem. biophys Res. Commun. *47* 81–87 (1972).

16 McCurdy P R., Sherman, A. S., Kamuzora, H., and Lehmann, H.. Globin synthesis in subjects doubly heterozygous for hemoglobin G Philadelphia and hemoglobins S or C. J Lab. clin. Med *85* 891–897 (1975).

17 Politis-Tsegos, C.; Lang, A., Stathopoulou, R., and Lehmann, H.. Is haemoglobin G_α Philadelphia linked to α-thalassaemia? Further evidence. Humangenetik *31* 67–74 (1976).

18 Kan, Y W and Nathan, G Mild thalassemia: the result of interactions of alpha- and beta-thalassemia genes. J clin. Invest. *49* 635–642 (1970).

19 Lehmann, H. and Lang, A.. Haemoglobin Q and thalassaemia. J sci. Soc. Thailand *1* 41–48 (1975).

Received: January 3, 1979
Accepted, February 15, 1979

Prof. H. Lehmann, Department of Biochemistry
Tennis Court Road, Cambridge
CB2 1QW (England)

The ratio of α^A to α^Q radioactivity was 2 1 in one son (III 1) somewhat lower in the other (III 2) From these data it appears that both siblings have inherited from the propositus the genes for α-thalassaemia and α^Q Philadelphia.

Discussion

The combined haematological and genetic information indicates that the propositus is a heterozygote for α^Q Philadelphia plus α-thalassaemia type 2 i.e. one gene for α-thalassaemia, and either two for β^o thalassaemia, or one for β^o and one for δβ-thalassaemia.

The presence of α-thalassaemia is supported by the fact that the proportion of α^Q-chains is a third of the total α-chains. It has been pointed out by *French and Lehmann* [2] and later by *McCurdy et al.* [16] and *Politis Tsegos et al* [17] that the proportion of more than 25% of this Hb in heterozygotes can be explained by its association with α-thalassaemia.

The mild degree of chain imbalance (α/non-α 1.38) in the propositus as compared with the β^o thalassaemia or even β^+ thalassaemia trait, in which the recorded α-non-α ratios are higher than 1 70 almost certainly results from the co-inherited α-thalassaemia. The co-inheritance of both α and β-thalassaemia and its clinical and haematological features have been described before [3 18, 19]

The nature of the β-thalassaemia in this case however is not clear The absence of β-chains at first suggests that it is of β^o-type. No data are available for the propositus mother but his father and two of his sons are carriers of a β-thalassaemia-like trait since their Hb F and Hb A_2 levels are high.

For the propositus, the differential diagnosis must be made between a homozygous β^o thalassaemia and for heterozygosity for a gene responsible for β^o-thalassaemia and one for δβ-thalassaemia or a Lepore-like condition. The low Hb A_2 level in the propositus is more compatible with δβ-thalassaemia but the Hb A_2 level in his father and his sons is unusually high and suggests that it represents a Lepore-like haemoglobin. The chemical analysis shows that the Hb A_2 consists of normal δ-chains. However this does not exclude a Lepore chain, if the δβ-crossing-over occurred after residue 126 This would result in what is chemically a δ-chain, but genetically a δ/β- or Lepore chain. This could possibly explain the high radioactivity incorporated in the region where δ-chains elute in the propositus bone marrow sample which amounted to 25% of total non-α-radioactivity (fig 2). This is a high value and δ-chain incorporation values obtained from thalassaemic bone marrow cells in our laboratory averaged no more than 10% of the total non-α-chain values It is unlikely that this peak is due to an abnormal β-chain since it was not detected in reticulocytes and mature red cells. Thus the 'Hb A_2 chain could be chemically a δ-chain but genetically a δβ-chain, with a relatively small amount of structural β-mRNA only being responsible for the C terminus. The difference between the levels of the δ-chain (low in the propositus and high in his first-degree relatives) would then have its explanation in the absence of interaction with any β-chain production in the propositus. These considerations are based on the very high 'Hb A_2 values found in the propositus first-degree

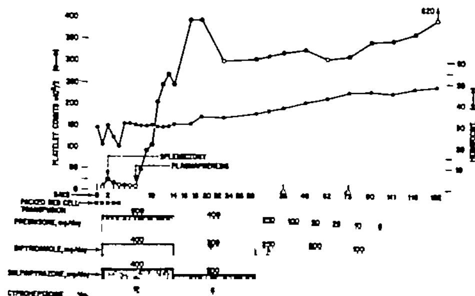


Fig. 1. Clinical course of patient with TTP. Dramatic improvement of the platelet count and hematocrit after plasmapheresis is illustrated.

pheral smear showed schistocytes, microspherocytes, and nucleated red blood cells. A bone marrow aspirate revealed hyperplasia of the megakaryocytes and erythroid elements. Urinalysis showed 1+ protein, 3–5 WBC/HPF and 1 3 RBC/HPF. BUN was 27 mg/dl, serum LDH 1,585 U, prothrombin time 29.8 sec (control 33.5 sec), partial thromboplastin time 29.8 sec (control 33.5 sec), fibrinogen 200 g/l. Fibrin degradation products were less than 40 μg/dl. LE cell test, immunofluorescent antinuclear antibodies, and direct Coombs' test were negative. Splenectomy and hysterotomy were performed on the 2nd hospital day. A slightly macerated fetus was delivered. The histology of the spleen showed intravascular hyaline thrombi. Postoperatively the hematocrit was 24.4%, and platelets were 18.0 × 10⁹/l. Dextran 75 was started immediately after surgery but was discontinued because of acute respiratory distress and heart failure. Fever, hemolysis, and thrombocytopenia persisted despite continuation of prednisone 300 mg/day, dipyridamole 400 mg/day, sulfinpyrazone 400 mg/day and cyproheptadine 12 mg/day. On the 7th hospital day plasma exchange was performed at the bedside utilizing the Haemonetics Model 30 Blood Cell Separator. A total of 3,560 ml of fresh frozen plasma was used during this procedure. 18 h after the procedure, the platelet count was 49.0 × 10⁹/liter and gradually rose to 220.0 × 10⁹/liter by the 12th hospital day. The hematocrit began to rise from 28 to 30% by the 3rd day after plasma exchange, as reticulocytes and schistocytes decreased. She was discharged on the 29th hospital day on corticosteroids and antiplatelet aggregating drugs. The dosage of these agents was gradually reduced, and they were discontinued by the 3rd month after her discharge. No evidence of TTP has returned during 15 months of follow-up observation.

Comment

TTP in association with pregnancy has been reported in 31 previous instances [5, 6, 18]. The series of 29 cases reviewed by *Schwartz and Brenner* [18] however contained only 8 cases in which preeclampsia-eclampsia was not present. Since the pentad of microangiopathic hemolysis,

Acta haemat. 62 112–116 (1979)

Thrombotic Thrombocytopenic Purpura in Early Pregnancy

Remission after Plasma Exchange

Chun Yang Murray Nussbaum and Hydow Park

Departments of Medicine and Pathology Martland Hospital Unit, College of Medicine and Dentistry of New Jersey New Jersey Medical School, Newark, N J

Key Words. Plasma exchange Pregnancy Thrombotic thrombocytopenic purpura

Abstract. A 24-year-old Chinese female developed fever fluctuating neurological and renal manifestations microangiopathic hemolytic anemia, and thrombocytopenia during the 11th week of her first pregnancy Therapy for thrombotic thrombocytopenic purpura (TTP) initiated during the 15th week of her pregnancy including corticosteroids, platelet aggregation inhibitors, hysterotomy and splenectomy was ineffective However dramatic improvement and remission occurred after plasma exchange of 3,560 ml was performed with the Haemonetics Model 30 Blood Cell Separator

Despite a relatively high incidence in women of child bearing age, thrombotic thrombocytopenic purpura (TTP) has rarely been reported during pregnancy [5 6 18] The patient presented developed the onset of fever hemolytic anemia, thrombocytopenia, and neurological and renal manifestations during the 11th week of gestation. The diagnosis of TTP was made during the 15th week. Remission was obtained only after plasma exchange was added to the treatment regimen which included high doses of corticosteroids platelet aggregation inhibitors, hysterotomy and splenectomy

Report of a Case

A 24-year-old Chinese female, in the 15th week of her first pregnancy was transferred to Martland Hospital with a diagnosis of probable TTP associated with pregnancy 4 weeks prior to admission, she noted intermittent nausea, vomiting, weakness, dizziness, easy bruisability and petechiae on her extremities. 5 days prior to admission, she had a transient syncopal episode, followed by expressive aphasia lasting for about 5 min. She was hospitalized elsewhere and was found to have a temperature of 39 °C, hematocrit 15%, platelet count 6.0×10^9/liter and 4+ proteinuria. She was started on prednisone 100 mg/day dipyridamole 400 mg/day folic acid 1 mg/day and was transferred to the Martland Hospital after transfusions with packed red blood cells.

On admission, she was alert. Temperature was 37.8 C; pulse 110/min, BP 110/70 mm Hg. Bilateral retinal hemorrhages were noted, and petechiae and ecchymoses were present on all extremities. Uterine gestational size of 14 weeks was estimated by ultrasonographic techniques. Hematocrit was 21 %, reticulocyte count 8%, and platelet count 6.0×10^9/liter The subsequent laboratory data and course are shown in figure 1. The peri-

let aggregating agents, splenectomy and a single plasma exchange has been in continuous remission for 15 months.

The striking increase in the number of remissions obtained with the use of blood or plasma exchanges, supports recent speculations that some cases of TTP and related disorders, such as the hemolytic-uremic syndrome, may be due to the presence of an unusual class of immune complexes which are active against vascular tissue and platelets [3 5] A deficiency of a normal plasma factor, concerned with inhibiting platelet aggregation and preventing microangiopathic hemolysis [19], has also been suggested in some cases [6 10] Some properties of TTP plasma have been determined [10] It induced *in vitro* aggregation of washed platelets from normal donors and from a patient in remission. The aggregating activity diminished as a function of time, when the TTP plasma was incubated with normal plasma at 37 C. *Remuzzi et al.* [15] have suggested that this factor stimulates vascular prostacyclin (PGI_2), a potent endogenous inhibitor of platelet aggregation.

Since remissions have also been obtained and maintained with multiple infusions of fresh frozen, stored or cryoprecipitate-free plasma in a few cases [6, 7 12, 16] it is suggested that plasma infusions be part of the initial therapy for TTP Plasma or whole blood exchange should be considered when the initial therapeutic modalities are unsuccessful.

References

1 Abramson, N Treatments for thrombotic thrombocytopenic purpura. Plasma, vincristine, hemodialysis and exchange transfusions. Letter. New Engl. J Med. 298 971–972 (1978).

2 Amorosi, E. L. and Karpatkin, S. Antiplatelet treatment of thrombotic thrombocytopenic purpura. Editorial. Ann. intern. Med. 86. 102 105 (1977).

3 Aster R. H. TTP New clues to the etiology of an enigmatic disease. Editorial. New Engl. J Med. 297 1400–1401 (1977).

4 Bukowski, R. M., Hewlett, J. S Harris, J W Hoffman, G. C., Bottle, J D., jr Silverblatt, E., and Yang, L Y Exchange transfusions in the treatment of thrombotic thrombocytopenic purpura. Semin. Hemat. 13 219–232 (1976).

5 Bukowski, R. M.; King, J W and Hewlett, J S. Plasmapheresis in the treatment of thrombotic thrombocytopenic purpura. Blood 50· 413–417 (1977).

6 Byrnes, J J and Khurana, M. Treatment of thrombotic thrombocytopenic purpura with plasma. New Engl. J Med. 297 1386–1389 (1977).

7 Byrnes, J J Sholtes, C. M., and White, P Plasma infusion in the treatment of thrombotic thrombocytopenic purpura, further experience. Blood 50: suppl. 1, p. 236 (1977).

8 Dekker A., O'Brien, M. E., and Cammarata, R. J The association of thrombotic thrombocytopenic purpura with systemic lupus erythrematosus. Am. J med. Sci. 267 243–249 (1974).

9 Fisher W B. Exchange transfusion in acute thrombotic thrombocytopenic purpura. Milit. Med. 142. 789–790 (1977).

10 Lian, E C. Y.; Byrnes, J J and Nunez, R. Pathogenesis of thrombotic thrombocytopenic purpura (TTP). Deficiency of plasma factor which inhibits platelet aggregation. Clin. Res. 26. 506A (1978).

11 Monnens, L. A. H. and Retera, R. J M. Thrombotic thrombocytopenic purpura in neonatal infant. J Pediat 71 118–123 (1967).

12 Okuno, T Plasmapheresis for thrombocytopenia. Letter Lancet i: 1095 (1978).

13 Pisciotta, A. V Garthwaite, T; Darin, J and Aster, R. H.: Treatment of thrombotic thrombocytopenic purpura by exchange transfusion. Am. J Hemat. 3 73–82 (1977).

14 Reiss, R.; Shah, V Kalter R., and Panillio, A. Plasmapheresis in thrombotic thrombocy-

Table I. Exchange transfusions in TTP. Summary of therapy in 29 patients

Number	Exchange	Steroids	Splenectomy	Platelet inhibitors	Other treatment	Number of remissions	References
3	+	0	0	0	0	2	[4 5]
12	+	+	0	0	androgen-1 heparin-1 plasma 1	9	[4, 5 11 13, 17]
7	+	+	+	0	0	2	[5, 8, 9 20]
5	+	+	+	+	Dextran-2 hemodialysis, heparin-1 vincristine 1 plasma-1	4	[1 6, 13 14]
2	+	+	0	+	hemodialysis, plasma-1	2	[13, 16]

Includes our case.

thrombocytopenia, fever neurological and renal manifestations can also be found in other disorders, including preeclampsia-eclampsia, the authors concluded that the diagnosis of TTP may have been inappropriate in many of these cases. In the case presented, symptoms suggesting TTP commenced during the 11th week of gestation. The presence of hyaline thrombi in the spleen supported this diagnosis

Dramatic improvement occurred in our patient only after a plasma exchange of 3.560 ml of fresh frozen plasma was added to the treatment regimen. To date, 31 patients with TTP treated with whole blood or plasma exchanges have been reported [1 4–6 8 9 11–14 16 17 20] Table I summarizes the various treatment modalities for our patient, and 28 reported cases, in which *complete details of therapy were provided.* 23 of these patients were exchanged with whole blood, 3 with plasma [5 12] and 3 with both [5 13 14] Multiple exchanges were required in 21 cases. 1 patient [14] developed an adverse hemolytic reaction during plasmapheresis, but subsequently responded to a whole blood exchange transfusion. 3 patients also received maintenance infusions of fresh frozen plasma [6, 12, 16]

Remissions, obtained in 19 patients, ranged from 5 months to over 13 years at the time of publication. 10 patients were reported to be in remission for over 1 year It is of interest that remissions were obtained without splenectomies in 13 of 17 patients (76%) 3 patients, including our own, were also pregnant at the time the diagnosis of TTP was made. Spontaneous abortions occurred at 23 weeks of gestation in one [5] and at 19 weeks in the other [6] In our patient, a macerated fetus was delivered by hysterotomy during the 15th week. The patient reported by *Bukowski et al.* [5] was treated only with multiple exchange transfusions of blood and plasma, and was in re*mission for 5 months at the time of the re*port. The case of *Byrnes and Khurana* [6] treated with steroids, splenectomy antiplatelet aggregating agents, multiple whole blood exchange transfusions, and plasma infusions, had been in remission for 14 months. Our patient, treated with steroids, antiplate-

Short Communications

Acta haemat. 62: 117–118 (1979)

Levamisole and Autoimmunity in Angioimmunoblastic Lymphadenopathy

Jean-Marie Andrieu, Pierre Youinou and Claire Herzog

Institut de recherches sur les maladies du sang, Hôpital Saint-Louis, Paris

Key Words. Angioimmunoblastic lymphadenopathy (AILD) · Levamisole · Autoimmunity

Abstract. A case of angioimmunoblastic lymphadenopathy (AILD) with important autoimmune symptoms disappearing under Levamisole therapy is reported. Since Levamisole is thought to have no direct effect on B cells, it is supposed that in AILD it regulates B lymphocyte activity via the restoration of impaired T cell functions.

Two reports concerning the treatment by Levamisole of angioimmunoblastic lymphadenopathy (AILD) have been published recently [1, 2]. The case we want to report is of particular interest, because it presented important autoimmune biological symptoms, which disappeared under Levamisole therapy. In October 1977 we admitted a 78-year-old man with enlarged generalized lymphadenopathies. 10 years previously he had an erythematous rash after a penicillin G treatment, followed by intermittent pruritus of the lower limbs and, 5 years later, by *the appearance of Raynaud's syndrome.* On examination, the patient was febrile and had generalized peripheral lymph node swellings. The spleen was not palpable, the liver was 4 cm below the right costal margin. Lymphangiography showed diffuse enlarged retroperitoneal lymph nodes. Chest X-ray was normal. Investigations revealed hemoglobin 10.6 g/dl, reticulocytes 140,000/μl, white cell count (WCC) 6,100/μl with 48% neutrophils, 3% lymphocytes, 15% monocytes and 33% eosinophils. Direct Coombs test was positive (IgG+C'), with a high titer of cold agglutinins (D = 1:4,096). We also detected a monoclonal cryoglobulinemia (IgM, λ = 3.5 mg/l) and a smooth muscle antibody high titer (D = 1:500). Bilirubin was 50 mg/l and alkaline phosphatase 644 U/l (normal: 80–250 U/l). WR and ANF were nonreactive. The percentage of E and EAC rosetting cells were, respectively 59 and 9% (check sample: 76 and 14%). The biopsy of an inguinal lymph node revealed typical features of AILD: diffuse proliferation of lymphocytes, eosinophils, immunoblasts, and plasma cells, and proliferation of small arborising vessels. The patient received Levamisole at a daily rate of 150 mg, 3 consecutive days every week,

topenic purpura. Letter Blood *51* 560–561 (1978).
15 Remuzzi, G Marchesi, D., Mecca, G Misiani, R., Livio, M., De Gaetano, G., and Donati, M B. Haemolytic-uraemic syndrome. Deficiency of plasma factor(s) regulating prostacyclin activity Lancet *II*. 871–872 (1978)
16 Rossi, E. C. and Del Greco, F. Treatments for thrombotic thrombocytopenic purpura. Plasma, vincristine, hemodialysis, and exchange transfusions. Letter New Engl. J Med. *298* 971–972 (1978).
17 Rubenstein, M. A. Kagan, E. M. MacGillivray M. H., Morliss, R., and Sacks, H. Unusual remission in a case of thrombotic thrombocytopenic purpura syndrome following fresh blood exchange transfusions. Ann. intern. Med. *51* 1409–1419 (1959)
18 Schwartz, M. L. and Brenner W E. The obfuscation of eclampsia by thrombotic thrombocytopenic purpura. Am. J Obstet. Gynec. *131* 18–24 (1978).
19 Upshaw J D Jr. Congenital deficiency of a factor in normal plasma that reverses microangiopathic hemolysis and thrombocytopenia. New Engl. J Med. *298* 1350–1352 (1978).
20 Wiles, S. A. and Sturgeon, P Thrombotic thrombocytopenic purpura. Review of the subject with a report of three cases in children. Pediatrics *17* 882–896 (1956).

Received. November 25, 1978
Accepted. January 25, 1979

Dr Chun Yang, MD Department of Medicine, Martland Hospital Unit, College of Medicine and Dentistry of New Jersey
New Jersey Medical School, Newark, N J (USA)

Acta haemat. 62: 119–120 (1979)

Prolonged Busulfan-Induced Remissions in Chronic Myeloid Leukemia

D. Benjamin, M. Djaldetti, Z. Mammon, M. Shaklai and J. Pinkhas

Department of Medicine 'D', Beilinson Medical Center, Department of Medicine 'B', Hasharon Hospital, Petah Tikva, and the Sackler School of Medicine, Tel Aviv University, Tel Aviv

Key Words. Busulfan · CML

Abstract. Two prolonged remissions were achieved in a patient with chronic myeloid leukemia by two short courses of busulfan treatment. The first remission lasted for 7 years, the second one lasts already 14 years. In the interval periods no treatment was administered.

Introduction

Prolonged busulfan-induced remission in chronic myeloid leukemia (CML) are rare [1–5]. We presently report a patient who entered into a 7 year remission, following a short course of busulfan [2]. At that stage a relapse occurred and another remission lasting already for 14 years was achieved, following a second short course of treatment with busulfan.

Case Report

The case history of this 54-year-old housewife was reported in detail in 1966 [2]. CML was diagnosed in 1957 and total dose of 185 mg busulfan, administered during 3½ months induced complete clinical and hematological remission which lasted for 7 years (Sept. 1957 to Sept. 1964). I January 1964, no Ph chromosome was detected. In September 1964 relapse occurred, Ph chromosome was identified [2] and total dose of 110 mg busulfan was administered during 3 weeks. The patient entered again into complete remission, lasting already for 14 years. Repeated chromosomal analyses did not reveal the presence of Ph chromosome.

Discussion

Galton and Spiers [4] reported Ph¹-positive CML patients who survived and entered into an unusually prolonged remission, lasting up to 14 years, and in whom the bone marrow became Ph¹-negative. The authors [4] suggested that in such patients the Ph¹-positive cells were unusually sensitive to busulfan and a marked reduction of the size of Ph¹-positive population might be instrumental for the release of the normal Ph¹-negative cells from a homeostatic sup-

Table I. Biological findings before and after Levamisole therapy

	Before	After 7 months
Hemoglobin, g/dl	10.6	13 1
Reticulocytes/µl	140,000	36 000
Bilirubin, mg/l	50	16
Cold agglutinins (titer)	4,096	64
Smooth muscle antibodies (titer)	500	0
E and EAC, %	59/9	66/30
Eosinophils/µl	2,000	1,000
Alkaline phosphatase, U/l	644	900

from November 1977 to May 1978 Under therapy the lymph nodes decreased and the hematological status progressively returned to normal. On May 15 there were no palpable peripheral lymph nodes, the size of the liver was the same the retroperitoneal lymph nodes had recovered a normal volume but had kept a pathological structure. Investigations showed hemoglobin 13 1 g/dl, reticulocytes, 3 600/µl, bilirubin 16 mg/l, wcc 6,500 µl with 75% neutrophils, 3% lymphocytes, 7% monocytes and 15% eosinophils. Cold agglutinins had gone down to 1 64 titer The smooth muscle antibodies had disappeared. E and EAC rosetting cells were, respectively 66 and 33% (check sample 68, 28%) The alkaline phosphatase was unchanged. Table I compares biological findings before and after 7 months of treatment. No adverse effects occurred under treatment and to date the patient is still treated with Levamisole

From an immunological point of view AILD is constituted by the association of autoimmune humoral symptoms and defect T cell functions. Up to now Levamisole was considered to have no direct effect upon B cells and to enhance T cell-mediated immunity [3] This case report may lead us to think that the normalization of B cell functions may be the result of the restoration of impaired T cell immunity

References

1 Bensa, J C., Favre, J., Martin, H., Sotto, J J and Schearer R.. Levamisole and immunoangioblastic lymphadenopathy Lancet i. 1081 (1976).
2 Ellegaard, J and Boesen, A. M.. Restoration of defective cellular immunity by Levamisole in a patient with immunoangioblastic lymphadenopathy Scand. J Haematol. 17 36–43 (1976)
3 Renoux, G Renoux, M., Teller M. N McMahon, A. M., and Guillaumin, J M.. Potentialisation of T-cell mediated immunity by Levamisole. Clin. exp. Immunol. 25 288–296 (1976).

Received. October 30, 1978
Accepted. November 15 1978

Dr J M. Andrieu, Chef de Clinique-Assistant,
Hôpital Saint Louis,
Institut de recherches sur les maladies du sang.
2, place du Dr Fournier
F-75010 Paris, (France)

Original Papers

Acta haemat. 62, 121 127 (1979)

Bone Marrow Transplantation for Severe Aplastic Anemia

A Report of 9 Cases

A M Marmont M T Van Lint G Avanzi G Reali R. Adami A Soldà P Strada M Barbanti M C. Mingari, O Soro G Grazi D Pedullà R Cerri, E Rossi D Giordano G Santini A Carella, M Risso R. Vimercati, G Piaggio M R. Raffo E. Librace V Vitale and A Bacigalupo

Department of Hematology Ospedale San Martino, and Blood Bank and Tissue Typing Laboratory Ospedale Galliera, Genova

Key Words. Bone marrow transplantation Aplastic anemia

Abstract. 9 patients with severe aplastic anemia (SAA) were treated with bone marrow transplantation (BMT). 5 were conditioned with cyclophosphamide and received and HLA-identical graft (4 patients) or a mismatched graft (1 patient) 1 rejected the graft on day 30 and died on day 34 during conditioning for a second transplant, 1 died on day 15 with acute and severe graft versus host disease (GvHD) in the absence of haemopoietic engraftment 3 are alive and complete chimeras at 1,069 490 and 332 days after transplantation. GvHD developed in 4 patients and was treated successfully in 3 with high dose *methylprednisolone and/or antilymphocytic* globulin (ALG). 4 patients were conditioned with ALG and received bone marrow from a haploidentical sibling or parent. 1 patient was refractory 3 patients showed evidence of hematologic reconstitution, but 2 of these required a second course of ALG 3 patients in this group are alive between 60 and 490 days 1 patient died on day 121 of HBSAg-negative acute hepatitis.

Bone marrow transplantation (BMT) has proven to be superior to conventional androgen-corticosteroid therapy in the management of severe aplastic anemia (SAA) [20] and encouraging results have been reported from several institutions [1 5 6, 15 18, 19] Nevertheless, major problems such as graft rejection and graft versus-host disease (GvHD) are still responsible for a 60% mortality rate [16] and need to be solved.

Because of these complications and because of the small number of patients with SAA who may have an HLA identical sibling, alternative treatments have been recently proposed including antilymphocytic globulin (ALG) with or without BMT [11, 14] and massive corticotherapy [2]

In the present report, 9 cases of severe aplastic anemia will be discussed together with the problems related to allogeneic identical or haploidentical BMT

pression, caused by the mass of the proliferating Ph[1]-positive cells.

The presently described patient is quite similar to the ones reported by *Galton and Spier* [4] thus adding a further proof to their important observations and explanation of the busulfan induced remissions in CML.

References

1 Cardinal, C. G.. Chronic granulocytic leukemia. Archs intern. Med. *136.* 305–313 (1976).
2 Djaldetti, M., Padeh, B Pinkhas, J and De Vries, A.. Prolonged remission in chronic myeloid leukemia after one course of busulfan. Blood *27* 103–109 (1966).
3 *Finney R. McDonald, G A., Balkie, A. G* and Douglas, A. S.. Chronic granulocytic leukemia with Ph' negative cells in bone marrow and a ten year remission after Busulfan hypoplasia. Br J Haemat. *23* 283–287 (1972).
4 Galton, D A. G and Spier A. S. D Progress in leukemias; in Progress in hematology pp. 343–405 (Grune & Stratton, New York 1971).
5 Weinberger A. Benjamin, D Douer D Barabash, G., Djaldetti, M., and Pinkhas, J A 13-years remission in chronic myeloid leukemia after a single course of Busulfan. Acta haemat. *59* 354–359 (1978)

Received. January 3 1979
Accepted. February 16, 1979

Prof. J Pinkhas, MD
Department of Medicine D
Beilinson Medical Center Petah Tikva (Israel)

Isolation procedures and gut decontamination. all patients were isolated in laminar air flow rooms (Matthews, USA), given oral antibiotics (gentamicin 40 mg every 6 h, nalidixic acid 500 mg every 6 h, colimycin 1,500,000 U every 6 h, miconazole 250 mg or nystatin 500,000 U every 6 h) and sterile food. Microbiologic monitoring was performed twice weekly

G HD prevention and treatment: methotrexate was given starting on day +1 according to the Seattle protocol [16]. Treatment with 6-methylprednisolone 20 mg/kg/day I. was started at the first clinical signs of GvHD and the dosage adjusted according to the patient's tolerance and response.

Results

Table I summarizes results and survival of the patients: 6 are alive between 60 and 1069 days from BMT 3 are complete chimeras, 2 have an autologous reconstitution, 1 is still being treated. 3 patients died. 1 of graft rejection, 1 of GvHD and 1 of acute hepatitis.

Case Reports

(1) T G., aged 25, male, was admitted after 60 days of conventional treatment for SAA given elsewhere and grafted after a large plasma exchange [9]. GvHD was initially treated with methylprednisolone and on day 120 with high dose horse ALG (400 mg/kg total dose): this second treatment resulted in complete resolution of skin and liver GvHD The patient is well and active 1,069 days after BMT

(2) M. M., aged 24, male, was admitted with 40-day history of SAA refractory to conventional treatment. He was given bone marrow from his HLA identical sister and had prompt engraftment; he rejected his graft on day 30 and died of sepsis on day 34 during conditioning for second transplant.

(3) T R., aged 17 male, was an uneventful transplant until day 84 when he developed interstitial pneumonitis: he was treated with Bactrim 4 tablets every 6 h and methylprednisolone 500 mg/

Number of BM cells 10^8/kg	Engraftment	Rejection	GvHD	Reconstitution	IP	Survival days	Cause of death or clinical performance
3.2	yes	no	II	chimera	no	> 1,069	alive, well
3	yes	yes		chimera	no	34	graft rejection
3.4	yes	–	I	chimera	yes	> 490	alive, well
3.5	yes	no	I	chimera	no	> 332	alive, chronic GvH
5.4	?		IV	?		15	acute GvHD
				part. autol			
3.6	no			compl. autol	–	> 526	alive, well
2.8	no			refractory			
				part. autol		121	acute hepatitis
2.2	no			refractory			
–			–	compl. autol		> 335	alive, well
2.8	no		–	refractory		> 60	alive, hospital

+2, +3 HALG = horse ALG RALG = rabbit ALG; part. autol = partial autologous (usually with less than 20 10^3 platelets/µl but self-sustained granulocyte counts) compl. autol = complete autologous reconstitution with self-sustained peripheral counts.

Materials and Methods

Patient data are outlined in table I All patients were diagnosed as having SAA according to current criteria [3] $< 5 \times 10^2$ granulocytes/μl, $< 20 \times 10^3$ platelets/μl < 1% reticulocytes and a hypoplastic marrow

Engraftment was monitored in 7 patients with chromosome studies (sex mismatched pairs) and in 2 patients with red blood cell phenotypes (ABO mismatched pairs) chimerism was shown, in 4 cases, to be complete with 100% markers of donor's origin both in blood and bone marrow

Partial autologous recovery was defined as a self-sustained granulocyte count at $2 \times 10^3/\mu$l, reticulocyte count of 1%, also in the absence of self sustained platelet levels, with initial evidence of hemopoietic restoration in the bone marrow preparations.

Complete autologous recovery was defined as self sustained counts of all three cell lines (granulocytes $> 2 \times 10^3/\mu$l platelets $> 80 \times 10^3/\mu$l reticulocytes 1% hematocrit 37%) with a regenerating bone marrow

Conditioning for BMT cyclophosphamide (CY) was given in patients 1–5 at the dosage of 50 mg/kg/day i.v on each of 4 consecutive days [13] In patients 1 and 2 it was preceded by the infusion of donor's buffy coat. In patient 5 it was followed by BMT and by the infusion of nonirradiated donor's buffy coat on days +1, +2, +3. HALG (horse ALG) (Berna, Switzerland) was given at the dosage of 40 mg/kg/day as an i.v drip in glucose 5% on days –6, –5 –4 –3, followed by a 2-day rest and BMT [14] Rabbit ALG (RALG; Rijks Instituut voor de Volksgezondheid, Bilthoven, The Netherlands) was administered at the dosage of 5 mg/kg/day as an i.v drip in glucose 5% on day –6 through day –3 followed by a 2 days rest and BMT

Transplantation. bone marrow aspirations were performed under general anesthesia from the posterior and anterior iliac cristae. 700–900 ml of marrow blood were then transferred to a Fenwal 1,000 ml bag and infused through a 15-μm filter, without further manipulation. Heparin (Boots, UK) 15,000 U in 20 ml of saline were employed to anticoagulate 500 ml of marrow blood.

Table I. Clinical data on 9 patients undergoing BMT with CY or ALG conditioning

Case	Age D	Age R	Sex D	Sex R	Etiology of SAA	Duration of disease	Transf n	ABO D R	HLA identity	Conditioning
1	42	24	M	M	idiop	60 days	20	A+O+	yes	BC-CY
2	27	22	F	M	p-hep	40 days	14	A+A+	yes	BC-CY
3	12	17	F	M	idiop	30 days	14	A+A+	yes	CY
4	14	11	M	F	p-pert	30 days	0	A+A+	yes	CY
5	40	7	M	F	fanconi	3 years	39	B+B+	no	CY/BC
6		24		M	idiop	180 days	27			HALG
	27		F					O+O+	no	RALG-BM
7	31	25	F	M	p-hep	120 days	49	A+O+	no	RALG-BM
										HALG
8	45	13	M	M	idiop	180 days	28	O+O+	no	HALG-BM
										RALG
9	36	7	M	F	p-hep	30 days	7	O+O–	no	HALG-BM

SAA = Severe aplastic anemia GvHD = graft-versus-host disease IP = interstitial pneumonia D = donor R = recipient idiop = idiopathic SAA p-hep = posthepatitis SAA p-pert = postpartum SAA BC-CY = donor's buffy coat on day –6 followed by CY (cyclophosphamide) 50 mg/kg/day on each of 4 consecutive days CY BC = CY as above followed by BMT and by the infusion of donor's buffy coat on day +1

were present before treatment. Detailed cytomorphologic data have been reported elsewhere [9].

Discussion

9 cases of SAA treated with BMT after cyclophosphamide or ALG conditioning are reported. 3 patients died 1 of acute GvHD on day +15 1 of graft rejection on day +34 and 1 of acute HBSAg-negative hepatitis on day +121 6 are alive between 60 and 1,069 days after BMT 3 are complete chimeras, 2 have autologous hematologic reconstitutions and 1 is still being treated (table I)

GvHD was associated with hemopoietic engraftment in 3 out of 4 cases In all it was treated with high dose methylprednisolone in addition to the methotrexate [16] protocol. 2 patients are free of GvHD (1 received additional ALG), 1 patient has mild chronic grade I skin GvHD (hyperpigmentation, no skin thickening), 1 patient died of GvHD The latter case developed clinical signs of GvHD on day 7 after receiving CY and the parent's marrow mismatched at the B locus for one antigen (see case report No. 5): treatment with high dose (20 mg/kg/day) 6-methylprednisolone did not ameliorate the GvHD and the patient died on day 15 with pathological evidence of skin-gut liver grade IV GvHD in the absence of hematopoietic engraftment. To this regard, *Storb* and coworkers [17] have reported acute GvHD with no engraftment in dogs grafted with mismatched marrow

ABO incompatibility was not a major obstacle for transplantation as already reported by other institutions [4] fractionated plasma exchange (patient 6) appeared to be better tolerated than the 20-hour continuous exchange of patient 1 Witebsky's substance was administered as reported elsewhere [10] Isolation was not broken during the plasma exchange, the procedure being carried out through an arteriovenous shunt with the patient in the laminar air flow room.

No takes were observed in 4 patients who were given ALG and haploidentical bone marrow in a recent review of the literature, 27% of patients receiving ALG and bone marrow had engraftments [11] but these proved to be transient with a median duration of 4 months. In our patients marrow reconstitutions were clearly autologous as proved by means of chromosome studies and red cell phenotypes. The reversal to normal blood and marrow parameters in 3 cases, as in a recent much larger series [15] would suggest that engraftment is not a prerequisite to ensure long-term survival as previously reported [11]

Three significant points concerning the pathogenesis and the treatment of SAA must be made at this stage. First, our results, both with bolus methylprednisolone [2] and with ALG, confirm *Speck*'s contention [15] that SAA is not uniformly a defect of the earliest hemopoietic progenitor cell, and that, at least in some cases, it may be an autoimmune condition not so distant from pure red cell anemia (PRCA), depending on the cellular target (stem cell in SAA, an unidentified erythroid precursor in PRCA) against which the immunologic reaction is mounted [7 8]

In the second place the occurrence of autologous hematologic reconstitutions would suggest, as already stated by others, that allogeneic BMT after CY conditioning may not be the best treatment for every patient with SAA, and should be attempted after careful selection. Recently the Seattle group has reported the overall incidence of

day Lv The radiological picture rapidly resolved and the patient's respiratory rate fell from 60 to 16/min in 4 days with an increase in the pO_2 from 57 to 99 mm Hg. Two episodes of granulocytopenia subsequently occurred and resolved under high dose methylprednisolone treatment. The patient is well and active 490 days after BMT

(4) M. L., aged 11 female, was transplanted untransfused and had a prompt engraftment. Chronic skin GvHD appeared on day 148 and is still being treated successfully with low dose methylprednisolone. The patient is active and well 332 days after BMT

(5) Z. F aged 7 female, admitted with the diagnosis of Fanconi-associated SAA treated elsewhere for 3 years with conventional therapy including androgens, corticoids and blood transfusions. The patient had an HLA identical sibling and the patient's father was homozygous for the A and B HLA loci. mixed lymphocyte cultures showed a relative response index [19] of 60 from recipient to sibling, of 38 between recipient and father of 1 between sibling and recipient, of 2.8 between father and recipient. The infusion of blood products from the father was thought to be responsible for the *high* RRI of the patient against both father and sibling· the RRI between sibling and patient was optimal, the RRI between father and patient suggested a lack of identity at the D locus. The HLA typing was as follows father A2,B18·Cw5 siblings A2 B14,18·Cw5 The identical sibling, aged 14 developed measles on the day of transplant with high temperature and mucositis. the father was therefore employed as a donor of both marrow and buffy coat on days +1, +2, +3 The patient developed acute and severe (grade IV) skin, liver and gut GvHD on day 7 and died on day 15 without evidence of hemopoietic engraftment.

(6) S. F., aged 24 male, after a first course of horse ALG resulting in a partial autologous reconstitution the patient was given rabbit ALG followed by the infusion of the HLA-mismatched sister's bone marrow The patient had a slow but steady hematologic reconstitution and is now 526 days after BMT well and active no evidence of engraftment could be obtained from cytogenetic studies.

(7) G E., aged 25 male, *was admitted with the* diagnosis of posthepatitis SAA refractory to androgens, corticoids and CY given elsewhere. He was treated with rabbit ALG and mismatched marrow from his sister with no engraftment and no hematologic reconstitution. He was therefore given a second course of ALG (horse ALG) to which he did respond, obtaining a granulocyte level of $2\times10^3/\mu l$, but still requiring once weekly platelet support. He was discharged and followed as an outpatient, but he was readmitted on day 115 with acute HBSAg-negative hepatitis of which he died on day 121

(8) G P., aged 13, male: the patient's history revealed hepatitis 21 months before the development of SAA he was given conventional treatment elsewhere. He was admitted with the clinical picture of a spinal section at C4 level (complete anesthesia from the neck down and paralysis at the four limbs) due to bleeding for severe thrombocytopenia. His peripheral counts and bone marrow were consistent with the diagnosis of SAA. The patient was given horse ALG and haploidentical bone marrow from his father· there was no engraftment and no hematologic reconstitution. After 2 months the patient was given rabbit ALG after 20 days from this course his granulocyte counts started to rise and at 60 days he was in complete hematologic reconstitution. The patient had a granulocyte count lower than $5\times10^2/\mu l$ for 71 days and was isolated in a laminar air flow room for 90 days: he received triple antibiotic therapy (gentamicin, carbenicillin and cephalothin) for 43 consecutive days he was given 200 U of random platelets and 16 U of packed RBC. This patient had intensive physical education while in the laminar air flow room he completely regained the use of all four limbs and is back to school doing well at 335 days after BMT

(9) D S., aged 7 female, admitted with the diagnosis of posthepatitis SAA. She was given horse ALG and her father's haploidentical bone marrow· there was no engraftment and no hematologic reconstitution. The patient is now receiving a second course of rabbit ALG *Bone marrow studies* were performed on material obtained by aspiration the aspirates were anticoagulated and, when particles were visible, isolated, assembled and gently but firmly spread on the slides; when no particles were found cytocentrifuge preparations were performed. In all cases typical inflammatory infiltrates [18] or aplastic granulomata [9]

severe aplastic anemia and acute leukemia. Nouv Revue fr Hémat. 21 133 (1979).

10 Marmont, A. M., Damasio, E. E., Bacigalupo, A., Giordano, D., Rossi, E., Reali, G Gay A., Dagna-Bricarelli, F Bruno, F Carella, A. M., and Santini, G. A to O bone marrow transplantation in severe aplastic anaemia, dynamics of blood group conversion and demonstration of early dyserythropoiesis in the engrafted marrow Br. J Haemat. 36. 511 518 (1977).

11 Mathé, G and Schwarzenberg, L. Treatment of bone marrow aplasia by bone marrow graft after conditioning with antilymphocytic globulin. Long-term results. Exp. Hematol., Copenh. 4 256–264 (1976).

12 Pederson-Bjegard, J Ernst, P and Nissen, N. L. Severe aplastic anemia with complete autologous marrow reconstitution following treatment with antilymphocytic globulin: report of case and review of the literature, Scand. J Haematol. 21 14–18 (1978).

13 Santos, G W Sensenbrenner L. L., Anderson, P N Burke, P J Klein, D L., Slavin, R. E., Schoter B and Borgaokar D. S. HLA-identical marrow transplantation in aplastic anemia, acute leukemia and lymphosarcoma employing cyclophosphamide. Transplant. Proc. 8 607–610 (1977).

14 Speck, B Gluckman, E., Haak, H. L., and van Rood, J J Treatment of aplastic anaemia by antilymphocytic globulin with and without bone marrow infusion. Lancet ii. 1145–1148 (1977).

15 Speck, B Cornu, P Nissen, C., Groff, P Weber W and Jeannet, M On the pathogenesis and treatment of aplastic anemia; in Baum and Ledney Experimental hematology today 1978, pp 143–149 (Springer, New York 1978).

16 Storb, R., Weiden, P L., Prentice, C. D., Buckner C. D Clift, R. A Einstein, A. B., Johnson, F L., Lerner K., Neiman, P E., Sanders, J E and Thomas, E. D. Aplastic anemia (AA) treated by allogeneic marrow transplantation. The Seattle experience. Transplant. Proc. IX. 181 185 (1977).

17 Weiden, P L., Storb, R., Graham, T C., Sale, G. E., and Thomas, D. E: Resistance to DLA-non identical marrow grafts in lethally irradiated dogs. Transplant. Proc. IX. 285–288 (1977).

18 T Velde, J and Haak, H. L. Aplastic anaemia. Histological investigation on metacrylate embedded bone marrow specimens. Correlation with survival after conventional treatment in 15 adult patients. Br J Haemat. 35 61–66 (1977).

19 Thomas, E. D., Fefer, A., Buckner C. D and Storb, R. Current status of bone marrow transplantation for aplastic anemia and acute leukemia. Blood 49 671–681 (1977)

20 UCLA Bone Marrow Transplant Team: Bone marrow transplantation in severe aplastic anemia. Lancet ii. 921–923 (1976).

Received. January 31, 1979
Accepted. May 9 1979

Alberto M Marmont, MD
Department of Haematology
Ospedale San Martino, Genova (Italy)

graft rejection and of GvHD in a large se ries of patients [16] from these data it may be possible to predict the likelihood of a pa tient for rejection (high relative response in dex, RRI low marrow dose) and the likeli-hood of a patient for GvHD (sex mismatch) We can, therefore, subdivide patients ac cording to prognostic indexes for transplantation and this may well change the plan of treatment of patients with SAA, since a 60% or more chance of early mortality has to be faced when grafting a patient who is refractory to random platelets, has a high RRI against his donor and has a sex mis-matched sibling. The contrary is true for pa tients who are referred untransfused without major infections in an early stage of their disease these patients would have a near to 100% chance of being cured with allogeneic BMT [unpubl. data] Alternative treatments with bolus methylprednisolone [2] or ALG with or without bone marrow infusion [14] may lead to complete autologous reconstitu tions in a considerable number of patients and, besides being the treatment of choice in patients without an HLA compatible sib-ling, may also be indicated in high risk pa tients even in the presence of potential com-patible donors.

Finally no indications have emerged from our patients in order to differentiate between treatment with ALG alone or fol-lowed by mismatched marrow infusion. Al-though it is believed that a transient take or microchimerism may provide an essential factor for the marrow microenvironment, or that the lymphocytes transfused with the marrow graft may influence favorably the disturbed interaction among lymphocyte subsets thought to be responsible for SAA [15] confirmation of this should still await the results of an ongoing randomized trial.

Acknowledgements

This work was supported by CNR grant No. 78.02845.96 The valuable assistance provided by the nursing staff is gratefully acknowledged.

References

1 ACS/NIH Bone Marrow Transplant Registry Bone marrow transplantation from histocompatible allogeneic donors for aplastic anemia. J Am. med. Ass. *236* 1131–1135 (1976).

2 Bacigalupo, A. Giordano D., Van Lint, M. T., Vimercati, R., and Marmont, A. M.. Bolus methylprednisolone in severe aplastic anemia. New Engl. J Med. *300*. 501–502 (1979).

3 Camitta, B. M. Thomas, E. D., Nathan, D. G., Santos, G., Gordon-Smith, E. C., Gale, R. P Rappaport, J M., and Storb, R.. Severe aplastic anemia. a prospective study of the effect of early marrow transplantation on acute mortality Blood *48* 63–70 (1976).

4 Gale, P G., Feig, S., Ho, W., Rippee, W and Sparkes, R.. ABO blood group system and bone marrow transplantation. Blood *50*. 185–194 (1977).

5 Gluckman, E., Bussel, A., Benbinam, M., Broquet, M. A., Schaison, J., Dausset, J et Bernard, J Traitement des aplasies de la moelle osseuse par la greffe de moelle osseuse allogénique. Nouv Presse méd. *4* 1177–1182 (1975)

6 Kolb, H. K. Wundisch, G F., Bender-Gotze, Ch., Spitzer I., Brehm, G., Rodt, H., v Lieven, H., Grosse-wilde, H., Albert, E. D Thiel, E., Ruppelt, W Balk, O and Thierfelder S.. Bone marrow transplantation in children with aplastic anemia and acute lymphatic leukemia. Blut *31* 343–346 (1975).

7 Marmont, A. M.. Pure red cell aplasia as an autoimmune receptor disease. Blood *49*. 155–156 (1977).

8 Marmont, A. M.. Serum antilymphocytaire pour le traitement des myélopathies aplasiques. Nouv Presse méd. *7* 2406 (1978).

9 Marmont, A. M.. Transplantation haemopoiesis. Morphological bone marrow studies after allogeneic marrow transplantation in man for

Material and Methods

During the period from October 1976 to September 1977 10 patients with aplastic anemia were seen in the hematology clinic of this institute. 6 of these 10 patients were categorized as having severe disease according to the criteria described by *Camitta et al.* [5] and were selected for FLI. In 1 of these cases (No. 4) the reticulocyte count was 1.8%, but when corrected for hemoglobin (Hb), it was less than 1%. In addition, 2 other cases (No. 3 and 7) were chosen for FLI because of their rapidly deteriorating condition even though they did not strictly satisfy the criteria of severe disease. 1 of the patients, however, declined the offer and has since been lost to follow-up. The rest of the 7 cases were given FLI. An informed consent was obtained from all of them after the procedure was fully explained. The presenting clinical picture of these 7 patients is shown in table I. Diagnosis of aplastic anemia in all these patients was made by peripheral blood findings (table II), bone marrow aspiration (table III) and bone marrow biopsy which was markedly hypocellular in all of them. Cases 1 and 4 gave history of chloramphenicol intake but its etiological role could not be ascertained as peripheral blood counts were not available prior to intake of chloramphenicol. Except in case 2, Ham's test and sucrose lysis test for paroxysmal nocturnal hemoglobinuria (PNH) were done before FLI in cases 4, 5, 6 and 7 and after FLI in cases 1 and 3. These tests were negative in all except in case 1 in which it was positive only temporarily for unexplained reasons. No evidence of pulmonary tuberculosis was found on chest X-ray in any of the cases. With the exception of case 5, all patients were treated for 4–10 weeks with repeated blood transfusions and high doses of oxy

Table I. Initial presenting data

Case No.	Age years	Sex	Fever/infection		Bleeding
1	12	M	+	gluteal abscess	nose
2	26	M	−	−	nose and gums
3	43	M	+	gluteal abscess	nose
4	16	F	+	−	nose and skin
5	42	M	+	−	nose
6	23	M	−	−	melena + skin
7	7	M	−	−	skin + nose

Table II. Hematological values

Case No.	On admission				1 day prior to fetal liver infusion			
	Hb, g/dl	Reticulocytes, %	Granulocytes/µl	Platelets/µl	Hb, g/dl	Reticulocytes, %	Granulocytes/µl	Platelets/µl
1	5.9	0.2	418	102,000	3.3	0.2	0	82,000
2	5.9	0.7	0	56,000	4.6	0.4	228	48,000
3	5.1	0.6	1120	48,000	4.4	0.1	300	56,000
4	5.7	0.1	540	60,000	3.8	1.8	126	38,000
5	7.7	0.8	0	26,000	3.8	0.2	168	76,000
6	8.6	0.8	396	46,000	4.1	0.2	120	58,000
7	4.3	1.0	660	80,000	5.7	0.8	900	40,000

Acta haemat. 62 128–136 (1979)

Fetal Liver Transplantation in Aplastic Anemia

Vinod Kansal Swaroop K Sood Anil K. Batra Gurcharan Adhar Anand K. Mahiya, Kiran Kucheria and Kamala Balakrishnan

Department of Medicine (Clinical Hematology), All-India Institute of Medical Sciences, New Delhi

Key Words. Aplastic anemia Fetal liver transplantation Temporary mixed lymphoid chimerism

Abstract. Fetal liver transplantation was attempted in 7 patients with aplastic anemia. 4 of these patients showed a partial response as evidenced by decrease in blood transfusion requirements and increase in the peripheral blood counts and hematopoietic cells in the bone marrow Bone marrow culture studies revealed evidence of a temporary mixed lymphoid chimerism (cases 1 and 3) While case 1 lived for 16 months case 3 is surviving at 17 months. None of the patients showed apparent graft versus-host disease. Increased incidence of infections was noticed. Possible causes for the same are discussed. 3 patients failed to respond Fetal liver transplant may be of therapeutic value in management of aplastic anemia.

Introduction

Fetal liver (FL) was used as a hematopoietic graft for the first time by *Uphoff* [18] She proposed that fetal hematopoietic tissue lacked immunological maturity and therefore might not produce graft versus-host disease (GVHD) under the circumstances where adult bone marrow was capable of doing so Indeed, she successfully precluded the secondary phase of irradiation syndrome by use of fetal hematopoietic tissue inoculation in mice exposed to lethal total body X irradiation. Subsequent animal studies [2] have provided further evidence that fetal hematopoietic tissue is a reasonable alternative to marrow transplantation particularly when a matched donor is not available. Recently some authors [1 3 11 13] have reported correction of severe combined immunodeficiency (SCID) by transplanting FL cells.

Following these reports, we have utilized fetal liver cell infusion (FLI) in 7 patients of aplastic anemia. There was evidence of temporary mixed lymphoid chimerism in 2 of these patients. 2 others showed a partial recovery while the remaining 3 failed to respond.

in heparinized culture medium (TC 199 + colchicine without phytohemagglutinin). The cultures were incubated for 3–4 h at 37 °C and harvested using the standard technique for chromosome preparations. 50 well spread metaphases were counted, out of which 15–20 were analyzed from each sample.

HLA Tissue Typing Studies

HLA antigens were studied before and after FLI in case 1 (following the second FLI) and in case 7. Antigens of HLA A and HLA B were identified according to the method used by *Terasaki and McClelland* [16].

Results

Of the 7 patients with aplastic anemia treated by FLI, 4 (cases 1, 2, 3, 7) showed variable but significant improvement as evidenced by partial amelioration of symptoms, decrease in bleeding episodes, decrease in blood transfusion requirements, rise in peripheral blood counts and reticulocytes. Also bone marrow showed increase in erythroid, myeloid and megakaryocytic series of cells with reduction in the lymphocytes and plasma cells (table III). These effects were observed between the 11th and 30th day of FLI.

Case 1 (fig. 1) had severe pancytopenia and required about 4–5 units of blood per week. Following FLI he showed hematological response by the 22nd day. This was, however, soon followed by repeated episodes of infections including malaria, recurrence of gluteal abscess, right-sided pneumonia and other febrile episodes of unknown etiology. Infections were associated with drop in

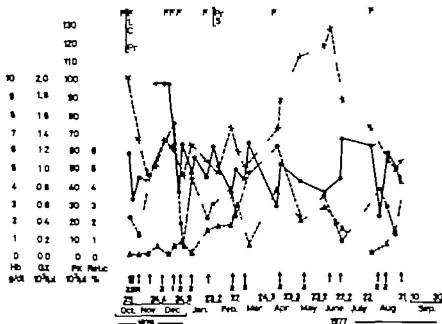


Fig. 1. Case 1. ● = Hemoglobin (Hb); O = granulocytes (G); × = platelets (P); ▲ = reticulocytes (Retic); ↑ = 1 unit of blood transfusion; ⇈ = 2 units of blood transfusion; F = fever; E = expired; FLC = fetal liver cells; Pr = prednisolone; PrS = prednisolone stopped.

metholone. The treatment with oxymetholone was discontinued and FLI was undertaken as there are several reports to indicate that oxymetholone is of little value in severe cases [7 12] and helps only mild to moderate disease [8] Besides, peripheral blood counts remained more or less the same and in most instances actually deteriorated in spite of high doses of oxymetholone. 2 patients who had mild disease were treated on conservative lines with oxymetholone and blood transfusions.

Procedure of Fetal Liver Infusion

Fetuses were obtained from patients in whom abortion had been induced by intra-amniotic prostaglandin administration. The FL was exposed and liver cells were aspirated with a syringe directly into heparinized minimum essential medium (MEM) under sterile conditions. Cell suspension was prepared and cell counts were made. Viability of the cells was checked with the Trypan blue dye exclusion test. Liver cell suspension was infused intravenously within 3 h of obtaining the fetus, if at least about 70% cells were viable. The number of FL cells infused in each patient are given in table IV

Apart from cases 4 and 5, all patients received 40 mg prednisolone daily for a period of 6-12 weeks starting from the day of FLI

Chromosomal Studies

Chromosomal studies to find evidence of chimerism were performed when the sex of the donor and recipient was different. These were done in case 3 and in case 1 in whom it was done after the second FLI.

Five drops of aspirated bone marrow were put

Table III. Marrow cellularity in percent

Case No	Pre-FLI cell pattern				Post FLI cell pattern				Days post-FLI
	myeloid	erythroid	megakaryocytic	nonhematopoietic	myeloid	erythroid	megakaryocytic	nonhematopoietic	
1	10	20	0	70	30	60	5	5	69
2	10	20	0	70	48	45	2	5	38
3	30	30	0	40	30	60	5	5	24
4	15	15	0	70	30	10	0	60	26
5	10	7	0	83	15	10	0	75	28
6	20	7	0	73	25	10	0	65	42
7	18	24	0	58	51	40	0	9	33

Table IV Fetal liver infusion

Case No.	Sex	Age of fetus weeks	Sex of fetus	Total number of FLC infused	Number of FL cells infused/kg body weight	Infection after fetal liver infusion	Final out come	Post FLI days	
1 I	M	16	male	10.5×10^7	3.55×10^6	yes		299	480
II	M	10	female	$5 7 \times 10^7$	1.8×10^6	yes	died	181	
2	M	12	not identified	$21 7 \times 10^7$	4.2×10^6	yes	died	120	
3	M	16	female	10.1×10^7	$1 6 \times 10^6$	yes	alive	500	
4	F	10	not identified	4.9×10^7	$1 1 \times 10^6$	nil	died	42	
5	M	13	male (twins)	41.8×10^7	6.85×10^6	nil	died	90	
6	M	14	male	$24 1 \times 10^7$	$4 66 \times 10^6$	yes	died	65	
7	M	11	male	2.3×10^7	$1 6 \times 10^6$	yes	died	130	

cyte and platelet counts remained very low and his blood transfusion requirements remained persistently high up to the 130th post-FLI day when he died of hemorrhage and infection. Last details are not given in figure 4.

Cases 4 5 and 6 did not show a satisfactory response and died on the 42nd, 90th and 65th post FLI days, respectively

Chromosomal studies for evidence of engraftment of the donor marrow were done on case 1 (after the second FLI) and on case 3 The preinfusion marrow culture of case 1 showed a fully male karyotype. 5 days post FLI the culture revealed cells both with XX(46) and XY(46) complements, their percentages in the 30 cells counted being 54 and 46 respectively The marrow culture was analyzed in case 3 on the 114th post FLI day It revealed both XX(46) and XY(46) types of chromosomes, their percentages in the 20 cells counted being 60 and 40 respectively The repeat chromosomal analysis of case 3 on the 239th post FLI day showed that the chromosomal pattern had returned to the XY pattern in all the 30 cells analyzed.

Pre- and post FLI HLA A and HLA B antigens were identified in case 1 (after the second FLI) and in case 7 In both cases, there was no change in HLA pattern. In case 1 however these studies were performed within 10 days of FLI and this period might not be sufficient to produce changes in HLA markers [4]

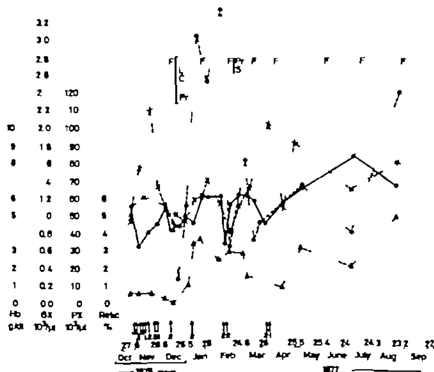


Fig. 3 Case 3. See figure 1 for details.

peripheral blood counts, but responded to appropriate antibiotics and supportive therapy with blood transfusions. On an average he required 2 units of blood per month. This beneficial effect lasted for 281 days when his blood transfusion requirements again increased and the hematological values dropped. A second FLI was given which produced a better hematological and clinical response. He had an episode of acute appendicitis following the 2nd FLI during the course of which he required 3 units of blood. On an average he was getting 1 unit of blood per month. This patient was lost to follow-up after his visit in December 1977. He was later reported to have expired on February 23rd, 1978 (480 days post FLI). Last details are not given in figure 1.

Case 2 (fig. 2) also showed an initial hematological improvement following FLI and from the 22nd to 52nd post FLI day he required only 2 units of blood transfusion. He was under observation up to the 81st post FLI day during which period he had a few episodes of infection associated with a drop in peripheral blood counts and rise in blood transfusion requirements. The infective episodes responded to appropriate antibiotic therapy. His bone marrow at this stage was cellular. This patient, however was lost to follow-up after discharge from hospital and it was learnt that he died after 120 days of the FLI.

Case 3 (fig. 3) showed even a better clinical and hematological response by the 11th day. From the 23rd post FLI day onwards he had episodes of fever each responding to ampicillin. His blood transfusion requirements, however gradually diminished and for a period of 5 months (April to August 1977) he required no blood transfusion. From September 1977 onwards his peripheral blood counts showed a gradual decline and he required blood transfusions once again. He is now alive at 500 days post FLI. He has, however, at present been readmitted for a possible second FLI. Last details are not included in figure 3.

Case 7 (fig. 4) showed hematological improvement on the 30th post FLI day with rise in hemoglobin to 8 g/dl and the erythroid and myeloid hyperplasia of the bone marrow (table III). A week later however he developed Pseudomonas infection of the ear associated with bleeding from the same site. His Hb dropped to 4.8 g/dl and he continued to be very sick. His hemoglobin, granulo-

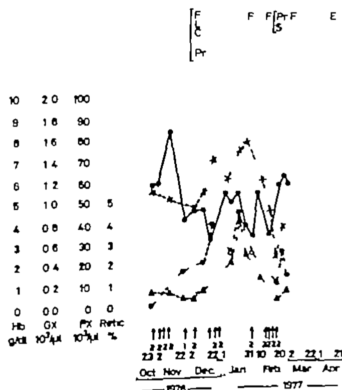


Fig. 2. Case 2. See figure 1 for details.

ceived transplants from female donors. 2 patients with the longest survival in our series were both males who had received FL cells from female donors and 1 of them was 43 years old.

The minimum dose of bone marrow of FLC for successful engraftment has not yet been defined. *Bortin et al.* [2] employing an FL or FLC + fetal thymus at doses of 6×10^7 cells/kg, demonstrated prompt recovery in lethally irradiated mice. We have been able to achieve engraftment by employing a much smaller dosage, for example 1.6×10^8 (case 3) and 1.8×10^4 (case 1 2nd FLI). In both the cases, however recovery was slow and incomplete. Further studies, therefore, are needed to define an optimum dose of FLI for treatment of aplastic anemia.

A high incidence of infection following FLI was observed in cases 1 2, 3 6 and 7 This may have been due to concomitant administration of prednisolone in these cases or due to continued low levels of granulocytes seen in them. A higher incidence of infection has been reported following bone marrow transplantation and has been attributed to relatively slow reconstitution of immunological functions following marrow grafting [10] No immunological studies were attempted in the cases under discussion.

Failure of response in cases 4 5 and 6 is not clear Possibility exists that they represent cases where aplasia results from microenvironment failure rather than a stem cell defect [9].

Bone marrow transplantation, though the best form of therapy for severe aplastic anemia at present, suffers from the disadvantage of enormous expense, difficulty of finding suitable HLA-matched donors, and a significantly high incidence of GVHD [5 6, 15 17] FLI thus may be of therapeutic value in management of aplastic anemia. The evidence of engraftment though temporary in 2 cases would suggest that this mode of therapy needs to be explored further The correct dosage of FL cells and need for concomitant therapy with oxymetholone and corticosteroids need to be worked out. Further studies are also required to understand the mechanism of beneficial effect and to find out the reasons for higher incidence of infection.

Acknowledgement

W extend our sincere thanks to Miss *Prema Rajagopalan*, Mr. *A K. Sehgal* and Mr *S C. Kasoch* for their technical help and Mr *V P Bhatnagar* for the typing of this manuscript. W are also grateful to the Department of Obstetrics and Gynaecology of this Institute for letting us utilize fetuses.

References

1 Bortin, M. M. and Rimm, A. A., Severe combined immunodeficiency disease. Characterization of the disease and results of transplantation a, b, c. Seventh report from the International bone marrow transplant registry J. Am. med. Ass. 238. 591–600 (1977).

2 Bortin, M. M., Rimm, A. A., Rose, W C., et al. Transplantation of hematopoietic and lymphoid cells in mice. Transplantation 21 331 336 (1976).

3 Buckley R. H., Whisnant, J K., Schiff, R. I., *et al.* Correction of severe combined immunodeficiency by fetal liver cells. New Engl. J Med 294 1076–1091 (1976).

4 Buckley R. H.: Transplantation of allogeneic bone marrow for correction of severe combined immunodeficiency in Perkins, an eight year perspective in human bone marrow transplantation. Symp Int. Soc. of Blood

Fig. 4. Case 7. See figure 1 for details.

Discussion

4 out of the 7 patients who received FLI showed partial response clinically and hematologically. Of these, 2 (cases 1 and 3) who had the longest survival also showed evidence of mixed lymphoid chimerism. The chimerism in these, however, appeared to be temporary because case 1 required FLI for a second time in 299 days, while in case 3 the cells with demonstrable female karyotypes derived from the FLI disappeared from bone marrow in 239 days. This would indicate that engraftment of donor cells may provide temporary benefit and in some cases may allow the time for subsequent regeneration of the patient's own bone marrow. In the absence of such regeneration, repeated fetal liver grafts may be necessary to ensure prolonged survival. Further studies are required to see if permanent engraftment of donor cells could be achieved.

In none of the patients under discussion has there been an apparent GVHD disease, as suggested by the absence of skin rashes, diarrhea and abnormal liver functions. The absence of GVHD may be due to the fact that fetal hematopoietic tissue lacks immunological maturity [18]. It may be pointed out, however, that GVHD has been observed following FLI in a case of SCID [3]. Furthermore, literature [1] indicates that GVHD was more severe when patients were older than 6 months and when males re-

ceived transplants from female donors. 2 patients with the longest survival in our series were both males who had received FL cells from female donors and 1 of them was 45 years old.

The minimum dose of bone marrow of FLC for successful engraftment has not yet been defined. *Bortin et al* [2] employing an FL or FLC + fetal thymus at doses of 6×10^7 cells/kg, demonstrated prompt recovery in lethally irradiated mice. We have been able to achieve engraftment by employing a much smaller dosage, for example 1.6×10^6 (case 3) and 1.8×10^6 (case 1 2nd FLI). In both the cases, however recovery was slow and incomplete. Further studies, therefore, are needed to define an optimum dose of FLI for treatment of aplastic anemia.

A high incidence of infection following FLI was observed in cases 1, 2, 3 6 and 7 This may have been due to concomitant administration of prednisolone in these cases or due to continued low levels of granulocytes seen in them. A higher incidence of infection has been reported following bone marrow transplantation and has been attributed to relatively slow reconstitution of immunological functions following marrow grafting [10] No immunological studies were attempted in the cases under discussion.

Failure of response in cases 4 5 and 6 is not clear Possibility exists that they represent cases where aplasia results from microenvironment failure rather than a stem cell defect [9]

Bone marrow transplantation, though the best form of therapy for severe aplastic anemia at present, suffers from the disadvantage of enormous expense, difficulty of finding suitable HLA-matched donors, and a significantly high incidence of GVHD [5 6 15 17] FLI thus may be of therapeutic value in management of aplastic anemia. The evidence of engraftment though temporary in 2 cases would suggest that this mode of therapy needs to be explored further The correct dosage of FL cells and need for concomitant therapy with oxymetholone and corticosteroids need to be worked out. Further studies are also required to understand the mechanism of beneficial effect and to find out the reasons for higher incidence of infection.

Acknowledgement

W extend our sincere thanks to Miss *Prema Rajagopalan*, Mr. *A. K. Sehgal* and Mr *S. C. Kaushik for their technical help and Mr V P Bhatnager* for the typing of this manuscript. We are also grateful to the Department of Obstetrics and Gynaecology of this Institute for letting us utilize fetuses.

References

1 Bortin, M M. and Rimm, A. A., Severe combined immunodeficiency disease. Characterization of the disease and results of transplantation a, b, c. Seventh report from the International bone marrow transplant registry J Am. med. Ass. 238 591–600 (1977).

2 Bortin, M. M., Rimm, A. A., Rose, W C., et al. Transplantation of hematopoietic and lymphoid cells in mice. Transplantation 21 331–336 (1976)

3 Buckley R. H. Whisnant, J K., Schiff, R. I., et al. Correction of severe combined immunodeficiency by fetal liver cells. New Engl. J Med. 294 1076–1091 (1976).

4 Buckley R. H.: Transplantation of allogenic bone marrow for correction of severe combined immunodeficiency in Perkins, an eight year perspective in human bone marrow transplantation Symp Int. Soc. of Blood

Transfusion and the American Association of Blood Transfusion and the American Association of Blood Banks, San Francisco 1976, pp. 79–81 (Gunthorp-Warren, Chicago 1977).

5 Camitta, B N., Rappoport, J M., Parkman, R., *et al* Selection of patients for bone marrow transplantation in severe aplastic anemia. Blood *45* 355–363 (1975)

6 Cline, M. J., Gale, R. P., Stiehm, E. R., *et al.* Bone marrow transplantation in man. Ann. intern. Med. *83* 691–708 (1975)

7 Davis, S. and Rubin, A. D.. Treatment and prognosis in aplastic anemia. Lancet *i.* 871–873 (1972).

8 Durate, L. Lopez-Sandoval, R. Esquivel, F and Sanchez Mendal, L. Androstane therapy of aplastic anemia. Acta haemat. *47* 140–145 (1972).

9 Editorial Mechanism in aplastic anemia. Lancet *ii.* 1179 (1976).

10 Fass, L. Ochs, H. D Thomas, E. D *et al.* Studies on immunological reactivity following syngenetic or allogenic marrow grafts in man. Transplantation *16* 630–640 (1973).

11 Githens, J H. Immunologic reconstitution with fetal tissue. New Engl. J Med. *294* 1116–1118 (1976).

12 Haak, H. L., Hartgrink-Groeneveld, G A. Guiot, H. E. L., *et al.* Acquired aplastic anemia in adults II. Conventional treatment: retrospective study in 40 patients. Acta haemat. *58.* 339–362 (1977).

13 Keightley R. G., Lawton, A. R., Cooper M. P., *et al.* Successful fetal liver transplantation in a child with severe combined immunodeficiency Lancet *ii.* 850–853 (1975).

14 Lynch, R. E., Williams, D M., Reading, J C, and Cartwright, G E. The prognosis in aplastic anemia. Blood *45* 517–528 (1975).

15 Storb, R., Thomas, D. E., Widen, P L., *et al.* Aplastic anemia treated by allogenic bone marrow transplantation, a report on 49 new cases from Seattle. Blood *48* 817–841 (1976).

16 Terasaki, P I. and McClelland, J D Microdroplet assay of human serum cytotoxine. Nature, Lond. *204* 998–1000 (1964).

17 UCLA Bone Marrow Transplantation Team: Bone marrow transplantation in severe aplastic anemia. Lancet *ii.* 921–923 (1976).

18 Uphoff, D E. Preclusion of secondary phase of irradiation syndrome by inoculation of fetal hematopoietic tissue following lethal body X irradiation. J natn. Cancer Inst. *20* 625–632 (1958).

Received. June 6, 1978
Accepted August 28, 1978

Dr Vinod Kansal, Lecturer in Medicine
(Clinical Hematology),
All-India Institute of Medical Sciences,
New Delhi, 110016 (India)

Acta haemat. 62: 137–142 (1979)

Acute Myelomonocytic Leukemia in Children

Possible Use of the Soft Agar Culture Technique in the Differentiation of Cellular Subtypes[1]

Y. Barak, A. Ma and N. A. Shore

Division of Hematology-Oncology, Childrens Hospital of Los Angeles, and Department of Pediatrics, University of Southern California School of Medicine, Los Angeles, Calif.

Key Words. Bone marrow cultures · Children · Myelomonocytic leukemia

Abstract. In 8 children with acute myelomonocytic leukemia (AMML), colony formation in soft agar cultures derived from bone marrow cells was studied in an attempt to differentiate the monocytic (Schilling) from the myelomonocytic (Naegeli) types. The children did not differ markedly in their clinical and morphological parameters. Three *in vitro* growth patterns were observed: markedly decreased or no growth in 4 cases, extensive growth of granulocytic colonies in 2 cases, and extensive growth of macrophage colonies in the remaining 2. It is suggested that the marrows presenting diminished or no growth patterns are presumably of acute myelogenous leukemia patients with a monocytic component. The excessive granulocytic or macrophage colony growth may be an *in vitro* indication for an *in vivo* proliferation of either granulocytic or monocytic leukemic cell lines, and therefore may represent the Naegeli or Schilling variants of AMML respectively. If these observations can be approved in a larger series of AMML patients, this approach can be valuable as another tool in the differential diagnosis of the subtypes of AMML in children.

Acute myelomonocytic leukemia (AMML), defined as leukemia involving the granulocytic and the monocytic cell lines, is rare in children, contributing only 7% to all childhood cases of acute leukemia [3]. It is generally accepted that by morphologic, cytochemical and muramidase assay methods there is no sharp demarcation between the more common myelomonocytic (Naegeli) and the rare 'pure' monocytic (Schilling) types of AMML. The morphologic aspects vary from mostly granulocytes with a few monocytes in the former to almost all monocytes in the latter [5, 6].

In children, significant variations in survival exist related to the morphology of the leukemic cell [3]. Thus, the delineation of specific forms of childhood AMML would be of potential clinical importance. The soft

[1] Financial support was received from the National Institutes of Health, Bethesda, USA, grant No. CA-11050.

Transfusion and the American Association of Blood Transfusion and the American Association of Blood Banks, San Francisco 1976, pp 79–81 (Gunthorp-Warren, Chicago 1977).
5 Camitta, B N Rappoport, J M, Parkman, R., *et al* Selection of patients for bone marrow transplantation in severe aplastic anemia. Blood *45* 355–363 (1975)
6 Cline, M. J Gale, R. P Stiehm, E. R., *et al* Bone marrow transplantation in man. Ann. intern. Med. *83* 691–708 (1975).
7 Davis, S. and Rubin, A. D Treatment and prognosis in aplastic anemia. Lancet *i.* 871–873 (1972).
8 Durate, L. Lopez-Sandoval, R., Esquivel, F and Sanchez Mendal, L. Androstane therapy of aplastic anemia. Acta haemat. *47* 140–145 (1972)
9 Editorial Mechanism in aplastic anemia. Lancet *ii.* 1179 (1976)
10 Fass, L. Ochs, H. D., Thomas, E. D *et al* Studies on immunological reactivity following syngenetic or allogenic marrow grafts in man. Transplantation *16* 630–640 (1973).
11 Githens, J H. Immunologic reconstitution with fetal tissue. New Engl. J Med. *294* 1116–1118 (1976)
12 Haak, H. L., Hartgrink-Groeneveld, G A. Guiot, H. E. L., *et al* Acquired aplastic anemia in adults. II. Conventional treatment: retrospective study in 40 patients. Acta haemat. *58*. 339–362 (1977).
13 Keightley R. G., Lawton, A. R., Cooper M. P *et al.* Successful fetal liver transplantation in a child with severe combined immunodeficiency Lancet *ii.* 850–853 (1975).
14 Lynch, R. E. Williams, D. M., Reading, J C, and Cartwright, G E. The prognosis in aplastic anemia. Blood *45* 517–528 (1975).
15 Storb, R., Thomas, D. E. Widen, P L, *et al.* Aplastic anemia treated by allogenic bone marrow transplantation, a report on 49 new cases from Seattle. Blood *48*. 817–841 (1976).
16 Terasaki, P L and McClelland, J D Microdroplet assay of human serum cytotoxins. Nature, Lond. *204* 998–1000 (1964).
17 UCLA Bone Marrow Transplantation Team: Bone marrow transplantation in severe aplastic anemia. Lancet *ii.* 921–923 (1976).
18 Uphoff, D E. Preclusion of secondary phase of irradiation syndrome by inoculation of fetal hematopoietic tissue following lethal body-X irradiation. J natn. Cancer Inst. 20 625–632 (1958).

Received. June 6, 1978
Accepted. August 28, 1978

Dr Vinod Kansal, Lecturer in Medicine (Clinical Hematology)
All India Institute of Medical Sciences,
New Delhi, 110016 (India)

Acta haemat. 62 137 142 (1979)

Acute Myelomonocytic Leukemia in Children

Possible Use of the Soft Agar Culture Technique in the Differentiation of Cellular Subtypes[1]

Y Barak, A Ma and N A Shore

Division of Hematology-Oncology Childrens Hospital of Los Angeles, and Department of Pediatrics, University of Southern California School of Medicine, Los Angeles, Calif.

Key Words. Bone marrow cultures Children Myelomonocytic leukemia

Abstract. In 8 children with acute myelomonocytic leukemia (AMML), colony formation in soft agar cultures derived from bone marrow cells was studied in an attempt to differentiate the monocytic (Schilling) from the myelomonocytic (Naegeli) types. The children did not differ markedly in their clinical and morphological parameters. Three *in vitro* growth patterns were observed. markedly decreased or no growth in 4 cases, extensive growth of granulocytic colonies in 2 cases, and extensive growth of macrophage colonies in the remaining _. It is suggested that the marrows presenting diminished or no growth patterns are presumably of acute myelogenous leukemia patients with a monocytic component. The excessive granulocytic or macrophage colony growth may be an *in vitro* indication for an *in vivo* proliferation of either granulocytic or monocytic leukemic cell lines, and therefore may represent the Naegeli or Schilling variants of AMML respectively If these observations can be approved in a larger series of AMML patients, this approach can be valuable as another tool in the differential diagnosis of the subtypes of AMML in children.

Acute myelomonocytic leukemia (AMML), defined as leukemia involving the granulocytic and the monocytic cell lines, is rare in children, contributing only 7 / to all childhood cases of acute leukemia [3] It is generally accepted that by morphologic, cytochemical and muramidase assay methods there is no sharp demarcation between the more common myelomonocytic (Naegeli) and the rare 'pure monocytic (Schilling) types of AMML. The morphologic aspects vary from mostly granulocytes with a few monocytes in the former to almost all monocytes in the latter [5 6]

In children, significant variations in survival exist related to the morphology of the leukemic cell [3] Thus, the delineation of specific forms of childhood AMML would be of potential clinical importance. The soft

[1] Financial support was received from the National Institutes of Health, Bethesda, USA, grant No. CA-11050.

agar culture system for growing colonies from human hematopoietic cells has become a useful tool for the investigation of cells in volved in the leukemic process [12, 13] The present study was conducted to exam ine the *in vitro* growth patterns of AMML marrows as a possible aid in differentiating the subtypes of this disease

Material and Methods

8 patients with AMML previously untreated or in relapse (off therapy for a minimum of 1 month) were studied (table I) The diagnosis of AMML was based on the presence in bone mar row and in peripheral blood of myelomonoblasts and an increase in the number of immature and mature monocytes. For further cytochemical clas- sification of the immature marrow cells into either the granulocytic or the monocytic series, staining for naphthol ASD chloracetate esterase (CAE) (granulocytic precursors) and, in 5 cases, for α-naphthyl acetate esterase (ANE) (monocytic precur sors) was employed [14] All patients except 1 (A. L.) were subsequently given combination ther apy of vincristine, cytoxan, cytosine-arabinoside, prednisone and thioguanine.

Colony formation by marrow cells was studied utilizing the method of *Pike and Robinson* [15] Bone marrow specimens were aspirated from the posterior iliac crest. The cell suspensions contain- ing 2 × 10⁶ cells/ml were plated over previously prepared underlayers of normal human peripheral blood leukocytes in plastic Petri dishes. The plates were incubated at 37 °C in a humidified incubator with a constant flow of 10% CO in air The counting of colonies consisting of 50 or more cells was done at day 12 with a dissecting microscope 30 colonies were removed from the ten plates of each marrow cultured. The cells were flattened on glass slides and stained with 0.45% aceto-orcein

Table I. Clinical and hematologic data

Patient	Age years	Sex	Clinical presentation	Monocytes in peripheral blood absolute number/mm³	Blasts in the marrow that was cultured %	Esterase reaction of marrow blasts		Clinical course
						CAE	ANE	
S.R	18	F	pallor bruising	64	19	pos	ND	CR, 13 m
K.M	10	F	pallor throat infection	0	75	pos.	ND	CR, 12 m
A.C.	14	F	bruising, throat infection	0	95	pos.	neg.	CR, 8 m
E.I	8 /	F	pallor ecchymoses	1 180	90	pos	ND	CR, 8 m
A.P	5½	M	hepatosplenomegaly 'preleukemia 6 mos.	3,528	65	pos.	neg.	NR, expired
N S	7	F	gum hypertrophy lymphadenopathy	11,500	37	pos.	neg.	CR, 10 m, relapsed NR, expired
R H	4 m	M	hepatosplenomegaly lymphadenopathy Dx. by liver biopsy	16,200	10	neg.	neg.	NR, expired
A.L.	4	M	Down's syndrome purpura, pallor	400	60	neg.	pos.	no Rx. expired

CR = Complete remission NR = no response ND = not done CAE = naphthol ASD chloracetate esterase ANE = α-naphthyl acetate esterase.

and Giemsa. Classification of the colonies into granulocytic and macrophage was based on morphologic criteria. In three cultures, confirmation of colony' cell type was obtained by staining for CAE and ANE activity

Results

Table I summarizes the clinical presentations and the hematological findings relevant to the study in the 8 patients. It shows that no separation into distinctive cytological subtypes of AMML was demonstrated. Moreover there was no correlation between either the presence or lack of monocytosis in the peripheral blood, or the classification of the marrow blasts into granulocytic or monocytic by the two specific esterase stains, and distinctive clinical courses.

Table II shows the number and the morphology of the colonies formed by each patient s marrow In contrast to the number of the colonies found regularly in our laboratory in cultures derived from normal marrows [2] the marrows of patients S. R, K. A., A. C. and E. I gave rise to either no growth or markedly decreased numbers of colonies. Most of the colonies that were obtained were small and granulocytic. Marrows of A. P and N S. gave rise to an extensive growth of colonies which were small, the majority of which consisted of granulocytes (fig. 1A, B).

A markedly increased number of colonies was also obtained from the marrows of R. H and A. L. These were normal-sized colonies of which the majority were composed of macrophages (fig. 1C, D). Staining

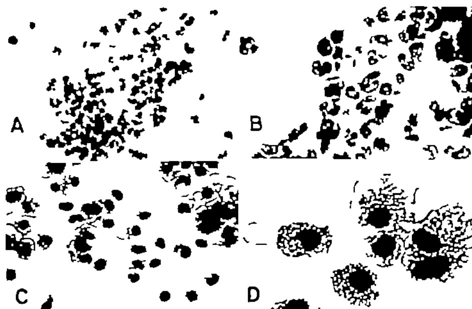


Fig. 1. Microscopic appearance of colony cells, at day 12 of incubation. A, B Granulocytes from the marrow culture of patient N. S. C, D Macrophages from the marrow culture of patient A. L. Aceto-orcein. A, C ×400; B, D ×1,000.

Table II. Colony formation by bone marrow cells

Patient	Number of colonies per 2×10^4 cells plated		Colony type, %	
	mean	range	granulocytic	macrophage
S. R	35	30–48	82	18
k. M	13	10–22	91	9
A. C	0	0	0	0
E. I	0	0	0	0
A. P	390	330–400	95	5
N. S.	360	304–392	95	5
R. H	283	272–297	15	85
A. L.	500	500	35	65
Normal[1]	50	24–200	95	5

for CAE and ANE performed on cells aspirated from the colonies of patients A. P., N. S. and A. L., was positive for CAE activity in A. P. and N. S. and positive for ANE activity in A. L. This confirmed the morphologic identification of granulocytic colonies from A. P. and N. S., and macrophage colonies from A. L.

Discussion

The present study has shown that by the parameters of clinical presentation, morphology and esterase staining, no distinctive patterns for specific variants of AMML were demonstrated. However, the colony growth and morphology in cultures derived from bone marrow cells of these patients, followed three patterns: markedly decreased to no growth, extensive growth of granulocytic colonies, or extensive growth of macrophage colonies.

Colony growth patterns in adult acute myelogenous leukemia (AML) and AMML have been described. In most studies, AML cells either failed to grow *in vitro*, or produced small abortive aggregates [2, 4–10]. In a few cases, however, extensive growth of colonies has been reported [12, 13]. In AMML, colony growth has been studied in a few instances. Defective growth was reported in cultures derived from 5 patients [10] and in another study of 4 patients excessive colony growth was obtained [7]. The colonies described in all the above studies were mostly granulocytic or were not defined morphologically. In juvenile chronic granulocytic leukemia, however, *Altman et al.* [1] have recently reported the production of exclusively monocytic colonies, in contrast with an increased granulocytic colony growth in the adult type of this disease.

Since recent studies using karyotypic markers have shown the leukemic origin of colonies derived from patients with leukemia [9], it is possible to assume that most colonies in cultures from leukemic patients arise from leukemic cells. Thus, from our results it would appear that in respect to the culture findings, one can separate this group of children with AMML into 3 subgroups. The diminished or no colony growth pattern reflects a type of *in vitro* granulopoiesis most commonly found in AML, and would presumably classify these patients in regard to their culture findings into AML with a monocytic reaction. As to the other two grwoth patterns, if the two subtypes of AMML in children are derived from two different cell lines, then the excessive growth patterns, if the two subtypes of ic colonies may represent either the true monocytic (Schilling) or the granulocytic (Naegeli) variants of AMML, respectively.

Another interpretation of these results may suggest that the two cell lines demon-

strated *in vitro* in AMML cases are actually the progeny of a common ancestor the granulocyte-monocyte progenitor The extent of differentiation of these progenitors into either the granulocytic or the monocytic cell lines is probably influenced by the *in vivo* ambient levels of colony-stimulating factor (CSF), an humoral factor that regulates granulopoiesis *in vitro* and probably plays a role in its regulation *in vivo* It has been postulated that elevation of CSF levels favors differentiation towards granulocytes, while low CSF levels favor differentiation towards monocytes [8] It is conceivable, therefore, that AMML in children is the result of a leukemic proliferation of one cell line, which differentiates into either the monocytic or granulocytic variant of AMML as influenced by CSF-related regulatory mechanisms.

The question whether these *in vitro* findings in AMML in children has any prognostic-therapeutic significance has not been answered in this small group of patients studied, as no correlation between the hematologic or clinical parameters and the three patterns of colony growth was shown However patients with decreased or no colony growth patterns, appeared to have had more favorable response to therapy and longer survival times than the AMML patients with increased colony growth treated by the same chemotherapeutic regimens. In this connection it is noteworthy that *Moore et al.* [11] were able to subdivide 108 cases of AML into four *in vitro* growth types. Although these growth subgroups did not differ markedly in hematologic parameters, they defined groups of patients with highly significant differences in response to therapy Also, *Spitzer et al* [15] have recently devised a simplified classification of AML patients based solely on leukemic proliferation *in vitro* 3 groups were recognized, and the complete remission rate was higher in the group with no growth and with small aggregates growth, than in the large aggregate growth group.

If further studies of more children with AMML will show significant correlation between *in vitro* growth subgroups and the clinical courses, the culture technique may be of value in a better subclassification and management of this disease.

References

1 Altman, A J Palmer, C. G and Baehner R. L. Juvenile 'chronic granulocytic' leukemia: panmyelopathy with prominent monocytic involvement and circulating monocyte colony forming cells Blood *45* 341 350 (1974).

2 Barak, Y Shore, N A Higgins, G. R., and Vadakan, V V Functional cellular maturation in cultures of human hematopoietic cells. Br J Haemat. *27* 537–549 (1974)

3 Fraumeni, J F Jr Manning, M. D and Mitus, W J Acute childhood leukemia Epidemiologic study by cell type of 1263 cases at the Childrens Cancer Research Foundation in Boston 1947–1965 J Natn. Cancer Inst *46*· 461–470 (1971).

4 Greenberg, P L., Nichols, W and Schrier S L. Granulopoiesis in acute myeloid leukemia and preleukemia New Engl. J Med. *284* 1225–1232 (1971).

5 Hayhoe, F G. J and Cawley J C. Acute leukemia cellular morphology cytochemistry and fine structure. Clin. Haematol. *1* 49–94 (1972).

6 Huguley C. M., Jr Acute granulocytic leukemia (AGL) and myelomonocytic leukemia. J Williams, Beutler Erslev and Rundles, Hematology (McGraw-Hill, New York 1972).

7 McCulloch, E. A. and Till, J E Regulatory mechanisms acting on hemopoietic stem cells. Am. J Path. *65* 601–619 (1971).

8 Metcalf, D and Moore, M. A. S Colony

Table II. Colony formation by bone marrow cells

Patient	Number of colonies per 2 × 10⁵ cells plated		Colony type, %	
	mean	range	granulocytic	macrophage
S. R.	35	30–48	82	18
K. M.	13	10–22	91	9
A. C.	0	0	0	0
E. I.	0	0	0	0
A. P.	390	330–400	95	5
N. S.	360	304–392	95	5
R. H.	283	272–297	15	85
A. L.	500	500	35	65
Normal[1]	50	24–200	95	5

for CAE and ANE performed on cells aspirated from the colonies of patients A. P., N. S. and A. L. was positive for CAE activity in A. P. and N. S. and positive for ANE activity in A. L. This confirmed the morphologic identification of granulocytic colonies from A. P. and N. S., and macrophage colonies from A. L.

Discussion

The present study has shown that by the parameters of clinical presentation, morphology and esterase staining, no distinctive patterns for specific variants of AMML were demonstrated. However, the colony growth and morphology in cultures derived from bone marrow cells of these patients, followed three patterns: markedly decreased to no growth, extensive growth of granulocytic colonies, or extensive growth of macrophage colonies.

Colony growth patterns in adult acute myelogenous leukemia (AML) and AMML have been described. In most studies, AML cells either failed to grow *in vitro* or produced small abortive aggregates [2, 4, 10]. In a few cases, however, extensive growth of colonies has been reported [12, 13]. In AMML, colony growth has been studied in a few instances. Defective growth was reported in cultures derived from 5 patients [10] and in another study of 4 patients excessive colony growth was obtained [7]. The colonies described in all the above studies were mostly granulocytic or were not defined morphologically. In juvenile chronic granulocytic leukemia, however, *Altman et al.* [1] have recently reported the production of exclusively monocytic colonies, in contrast with an increased granulocytic colony growth in the adult type of this disease.

Since recent studies using karyotypic markers have shown the leukemic origin of colonies derived from patients with leukemia [9], it is possible to assume that most colonies in cultures from leukemic patients arise from leukemic cells. Thus, from our results it would appear that in respect to the culture findings, one can separate this group of children with AMML into 3 subgroups. The diminished or no colony growth pattern reflects a type of *in vitro* granulopoiesis most commonly found in AML, and would presumably classify these patients in regard to their culture findings into AML with a monocytic reaction. As to the other two grwoth patterns, if the two subtypes of AMML in children are derived from two different cell lines, then the excessive growth patterns, if the two subtypes of ic colonies may represent either the true monocytic (Schilling) or the granulocytic (Naegeli) variants of AMML, respectively.

Another interpretation of these results may suggest that the two cell lines demon-

Acta haemat. 62: 143–147 (1979)

Terminal Deoxynucleotidyl Transferase Activity and B Cell Markers in Chronic Myelogenous Leukemia Blast Crisis

R. Sasaki, F. Takaku, S. Sakamoto and Y. Kanoh

Department of Medicine, Jichi Medical School, Kawachi, Tochigi Prefecture

Key Words. Blast crisis · Chronic myelogenous leukemia · Lymphoid precursor cell · Stem cell · Terminal deoxynucleotidyl transferase · Vincristine and prednisolone

Abstract. High terminal deoxynucleotidyl transferase activities were found in blast cells having B cell markers in 3 patients suffering from chronic myelogenous leukemia. The blast cells from these patients were lymphoblastic in appearance. Two of these cases were treated with vincristine and prednisolone. They responded well to this therapy.

Introduction

High levels of terminal deoxynucleotidyl transferase (TdT) activity have been detected in leukemic cells from most patients suffering from acute lymphoblastic leukemia (ALL) [1, 2] and from some cases afflicted with chronic myelogenous leukemia (CML) in blast crisis [3, 4]. This study presents the findings concerning 3 cases of CML in blast crisis where the blast cells had B cell markers and high TdT activity.

Materials and Methods

Chemicals

Poly-dA was obtained from P-L Biochemicals, Inc., Milwaukee, Wisc. ^{3}H-dGTP (1 Ci/mmol) was purchased from the Radiochemical Center, Amersham. Triton X 100 was a product of Shell Oil Co., New York, N.Y. All chemicals were of reagent grade.

Preparation of Cell Extract

Nucleated cells were separated from bone marrow aspirate by using a dextran sodium metrizoate solution [5] and were suspended at 1×10^8 cells/ml in sucrose TKM buffer (50 mM Tris-HCl, pH 7.6, 25 mM KCl, 5 mM $MgCl_2$, 5 mM dithiothreitol, 0.15 M sucrose, 100 µg/ml phenylmethylsulfonylfluoride and 0.1 mg/ml bovine serum albumin). The suspension was subjected to four cycles of rapid freezing and thawing. Then, Triton X 100 was added to the suspension at a concentration of 0.5% (v/v). The suspension was stirred for 15 min and centrifuged at 100,000 *g* for 60 min. The supernatant was used for the enzyme assay.

Enzyme Assay

The reaction mixture for TdT assay contained 50 mM Tris-HCl at pH 7.5, 30 mM KCl, 0.5 mM $MnCl_2$, 5 mM dithiothreitol, 10 µg bovine serum albumin, 0.5 units poly-dA and 1 µCi ^{3}H-dGTP. Poly-dA was used as the primer for the TdT assay

stimulation factor (CSF) in Haemopoietic cells (North-Holland, Amsterdam 1971).

9 Moore, M A. S. and Metcalf D Cytogenetic analysis of human acute and chronic myeloid leukemic cells cloned in agar culture Int. J Cancer *11* 143–152 (1973).

10 Moore, M A. S. Williams, N and Metcalf D., *In vitro* colony formation by normal and leukemic hematopoietic cells. characterization of the colony forming cells. J natn. Cancer Inst. *50* 603–623 (1973)

11 Moore, M. A. S. Spitzer G Williams, N., Metcalf D and Buckley J Agar culture studies in 127 cases of untreated acute leukemia the prognostic value of reclassification of leukemia according to *in vitro* growth characteristics. Blood *44* 1–18 (1974)

12 Paran, M. Sachs, L. Barak, Y and Resnitzky P *In vitro* induction of granulocyte differentiation in hematopoietic cells from leukemic and non leukemic patients. Proc. natn. Acad. Sci. USA *67* 1542–1549 (1970).

13 Pike, B L. and Robinson, W A. Human bone marrow colony growth in agar-gel. J cell Physiol. *76* 77–84 (1970)

14 Rozenszajn, L., Leibovitch, M Shoham, D. and Epstein, J The esterase activity in megaloblasts, leukemic and normal hematopoietic cells. Br J Haemat. *14* 605–610 (1968).

15 Spitzer G Dicke, K. A., Gehan, E. A., Smith, T., McCredie, K. B. Barlogie, B and Freireich, E. J.. A simplified *in vitro* classification for prognosis in adult acute leukemia. the application of *in vitro* results in remission-predictive models. Blood *48* 795–807 (1976).

Received January 1 1979
Accepted March 9 1979

Y Barak, MD Department of Pediatrics, Kaplan Hospital, Rehovot (Israel)

been well controlled either with busulfan or dibromomannitol until blast crisis occurred.

Results

Characterization and TdT Activity of Blasts

Blast cells in the present cases showed the morphological appearance of small to medium sized lymphoid cells. High TdT activity was detected in blast cells from all 3 of these patients, as shown in table II. Determination of the TdT activity in bone marrow cells at the time of blast crisis was not carried out in case 1 because of the inability to obtain enough material for the assay. However the TdT activity in blast cells from cerebrospinal fluid (CSF) was determined when the patient had a meningeal relapse 3 months after the onset of blast crisis and was found to be high.

The addition of deoxyribonucleoside triphosphates to the reaction mixture suppressed the TdT activity in case 2 and case 3. This suppression was more marked when three – rather than one or two – kinds of deoxynucleoside triphosphates were added. Furthermore, the addition of 5 mM N-ethylmaleimide inhibited more than 90% of the activity (data not shown).

The results of the analysis of the surface markers of blast cells in these CML cases in blast crisis are summarized in table II. It can be seen that 97% of the blast cells in CSF from case 1 formed EAC rosettes and 34% of the blasts possessed surface immunoglobulins. No E rosette-forming cells were detected. EAC rosette-forming cells composed 90% of the blast cells from case 2 and 30% of the blast cells from this patient possessed surface immunoglobulins. In case 3 EAC rosette forming cells composed 50% of the blast cells. The percentage of cells destroyed by the anti-Ia-like serum was 86%, while 88.2% were killed by the anti-B cell serum and 31% by the anti-T cell serum.

Response to Vincristine and Prednisolone Therapy

As shown in table I combination therapy with vincristine and prednisolone was given to cases 1 and 2. Complete remission was induced in case 1 while partial remission was achieved in case 2. Vincristine and prednisolone were not administered to case 3.

Discussion

This report presents data on 3 patients with CML in blast crisis and whose blast cells contained high levels of TdT activity. Analysis of the surface markers of the blast

Table II. TdT activity and cell surface markers of patients' blast cells

Case	Source of cells	TdT activity nmol 10⁸ cells	Rosette-forming cells E, %	Rosette-forming cells EAC, %	SmIg %	Reactivity of cells with, % anti-Ia	anti-B	anti-T
1	CSF	0.9	0	97	34			
2	Bone marrow	11.7	3	90	30			
3	Peripheral blood	13.6		50		86	88.2	31

in order to lessen the effect of nuclease. The reaction for the TdT assay was carried out for 20 min at 37 C. The reactions were terminated by cooling the reaction tubes in an ice bath. Aliquots were placed on Whatman GF/C glass fiber discs and processed according to the method of *Chang and Bollum* [6] The samples were counted in a toluene scintillator using a liquid scintillation spectrometer The background radioactivity was subtracted from the measured activity The TdT activity was expressed as nmoles of ^{3}H-dGMP incorporated per 1×10^{8} nucleated cells in 20 min. The TdT activity in normal human bone marrow cells was less than 0 09 nmol/10^{8} nucleated cells.

Analysis of Cell Surface Markers

Spontaneous rosette formation with sheep erythrocytes was used as the T cell marker indicator Surface immunoglobulin presence and rosette formation with erythrocytes sensitized with 19S antibody and complement were used to identify the B cells. Studies on the destruction of blast cells by anti T cell anti-B cell and anti-Ia sera were carried out by Dr *Kondo* at Kanazawa Medical University These anti-T and anti-B cell sera were obtained by immunizing rabbits with Molt-4F (T cell line) and PLKM (B cell line) cells. The specificity of these antisera was confirmed by their cytotoxicity to the various T and B cell lines [7]. Anti-Ia-like serum was prepared from the serum of pregnant women by Dr *Tsuji* of the Department of Medicine at Tokai University

Patients

The clinical and laboratory findings on these patients with CML in blast crisis and whose blast cells had elevated TdT activity and B cell markers are summarized in table I When the patients were first seen, they all had splenomegaly leukocytosis with low neutrophil alkaline phosphatase activity and hyperplastic bone marrow all of which are compatible with the diagnosis of the chronic phase of CML. Bone marrow cells from case 1 and peripheral blood cells from case 3 possessed the Ph[1] chromosome, but this chromosomal abnormality was not detected in case 2. These patients had

Table I Clinical and laboratory findings in patients with CML in blast crisis and whose blast cells had high TdT activity and B cell markers

Data	Case 1	Case 2	Case 3
Age, years and sex	27 M	38 M	32 M
Peripheral blood			
Hemoglobin, g/100 ml	10 4		10 0
Platelet count (+ 1,000)	364	200	29
White cell count (× 1,000)	20	69	300
Blast cells, %	2	54	53
Bone marrow			
Blast cells, %	87	83	dry tap
Ph chromosome	(+)	(−)	(+)
Clinical course			
Duration of chronic phase, years	7	4	2
Survival in blast crisis, months	6	7	2
Treatment	VP	VP CTN 6MP	N
Response	CR	PR	NR

VP = Vincristine-prednisolone CTN = cyclophosphamide N = neocarcinostatine.

been well controlled either with busulfan or dibromomannitol until blast crisis occurred.

Results

Characterization and TdT Activity of Blasts

Blast cells in the present cases showed the morphological appearance of small to medium sized lymphoid cells. High TdT activity was detected in blast cells from all 3 of these patients, as shown in table II. Determination of the TdT activity in bone marrow cells at the time of blast crises was not carried out in case 1 because of the inability to obtain enough material for the assay. However the TdT activity in blast cells from cerebrospinal fluid (CSF) was determined when the patient had a meningeal relapse 3 months after the onset of blast crisis and was found to be high.

The addition of deoxyribonucleoside triphosphates to the reaction mixture suppressed the TdT activity in case 2 and case 3. This suppression was more marked when three – rather than one or two – kinds of deoxynucleoside triphosphates were added. Furthermore, the addition of 5 mM N-ethylmaleimide inhibited more than 90% of the activity (data not shown).

The results of the analysis of the surface markers of blast cells in these CML cases in blast crisis are summarized in table II. It can be seen that 97% of the blast cells in CSF from case 1 formed EAC rosettes and 34% of the blasts possessed surface immunoglobulins. No E rosette-forming cells were detected. EAC rosette-forming cells composed 90% of the blast cells from case 2 and 30% of the blast cells from this patient possessed surface immunoglobulins. In case 3 EAC rosette-forming cells composed 50% of the blast cells. The percentage of cells destroyed by the anti-Ia-like serum was 86%, while 88.2% were killed by the anti-B cell serum and 31% by the anti-T cell serum.

Response to Vincristine and Prednisolone Therapy

As shown in table I combination therapy with vincristine and prednisolone was given to cases 1 and 2. Complete remission was induced in case 1 while partial remission was achieved in case 2. Vincristine and prednisolone were not administered to case 3.

Discussion

This report presents data on 3 patients with CML in blast crisis and whose blast cells contained high levels of TdT activity. Analysis of the surface markers of the blast

Table II. TdT activity and cell surface markers of patients' blast cells

Case	Source of cells	TdT activity nmol/10^8 cells	Rosette-forming cells E, %	Rosette-forming cells EAC, %	SmIg %	Reactivity of cells with, % anti-Ia	anti-B	anti-T
1	CSF	0.9	0	97	34			
2	Bone marrow	11.7	3	90	30	–		
3	Peripheral blood	11.6	2	50		86	88.2	31

cells of the present 3 cases revealed a predominance of B cell markers Therefore, the blast cells in these patients may represent putative bipotential lymphoid precursor cells having the capacity to differentiate into both T and B lymphocytes *Davis* [9] suggested that continued differentiation of lymphoid stem cells might result in the formation of cells capable of expressing both T and B cell markers at some point before functional dichotomy occurs

Recently the presence of high TdT activity in leukemic cells from a patient having acute undifferentiated leukemia [10] and in peroxidase-positive leukemic cells from 2 cases of acute myelogenous leukemia [11] and from 2 cases of myeloid blast crisis of CML [12] was reported. Furthermore, very recently an ALL patient was found to have high TdT activity in blasts seeming to possess B cell markers [13] These reports suggest that TdT is not necessarily a marker enzyme of T cell precursors and this enzyme activity is already present at the stage of pluripotent stem cells.

Recently much interest has been directed toward the target cells from which blast crisis of CML develops *Boggs* [8] suggested that CML might be a clonal disease of a pluripotent stem cell capable of lymphoid differentiation and that some of the cases of blast crisis might actually undergo lymphoblastic conversion. *Janossy et al* [14] reported 5 cases of CML in blast crisis in which the blast cells carried the Ph[1] chromosome and reacted with an antiserum specific for acute lymphoid leukemia cells of non T non B type They suggested that the aberration of the Ph chromosome in blast crisis of CML might take place in an undifferentiated pluripotent stem cell which is the common precursor of prelymphoid and myeloid cells [14] It is speculated that, in some cases of CML, blast crisis may induc reversion of leukemia myeloid cells to leukemic pluripotent stem cells.

Acknowledgements

Grateful acknowledgment is made to Dr . *Oono* University of Nagoya, and Dr *S Kan* Kanazawa Medical University for their provisi of patients clinical data, as well as the data of the cell surface markers of blasts from their patients. Their supply of the materials for the determination of the enzyme activity is also sincere appreciated.

References

1 Colman, M S Greenwood, M. F., Hutton, J Bollum, F J Lamplin, B., and Holland, P Serial observations on terminal deoxynucleoi dyl transferase activity and lymphoblast su face markers in acute lymphoblastic leukemi Cancer Res. *36* 120–127 (1976).

2 McCaffery R. Harrison, T A. Parkman, R and Baltimore, D Terminal deoxynucleotid transferase activity in human leukemic cel and in normal human thymocytes. New Eng J Med. *292* 775–780 (1975).

3 Sarin, P S Anderson, P N and Gallo, R C. Terminal deoxynucleotidyl transferase ac tivity in human blood leukocytes and lym phoblast cell lines high levels in lymphobla cell lines and in blast cells of some patient with chronic myelogenous leukemia in acut phase. Blood *47* 11–20 (1976)

4 Sarin, P A. and Gallo, R. C Terminal deoxy nucleotidyl transferase in chronic myelogen ous leukemia. J biol Chem *249* 8051–805 (1974).

5 Bøyum, A. Separation of white blood cells Nature, Lond. *204* 793–794 (1964).

6 Chang, L. M S. and Bollum, F J Low mo lecular weight deoxyribonucleic acid polymer ase in mammalian cells. J biol. Chem. *246* 5835–5837 (1971)

7 Shimura, S Osaka, Y Sogal, S and Konda, S Antisera to human T and B cells; in Methods in immunology vol. VI, pp 1121 1128 (Japanese Society of Immunology Kanaza 1977).
8 Boggs, D R.. Hematopoietic stem cell theory in relation to possible lymphoblastic conversion of chronic myeloid leukemia. Blood *44* 449-453 (1974).
9 Davis, S Hypothesis: differentiation of the human lymphoid system based on cell surface markers. Blood *45* 871–880 (1975)
10 Marcus, S K., Smith, S. W Jarowski, C I and Modak, M J Terminal deoxynucleotidyl transferase activity in acut undifferentiated leukemia. Biochem. biophys. Res Commun *70* 37–44 (1976).
11 Srivastava, B I. S., Khan, S. A and Henderson, E. S High terminal deoxynucleotidyl transferase activity in acute myelogenous leukemia. Cancer Res. *36* 3847–3850 (1976)
12 Srivastava, B. I. S Khan, S A Minowada, J Gomez, G A., and Rakowski, I Terminal deoxynucleotidyl transferase activity in blastic phase of chronic myelogenous leukemia. Cancer Res *37* 3612 3618 (1977).
13 Shaw M. T Dwyer J M Allaudeen, H. S., and Weitzman, H. A.. Terminal deoxynucleotidyl transferase activity in B-cell acute lymphocytic leukemia. Blood *51* 181 187 (1978)
14 Janossy G Greaves, M F Revesz, T Lister, T A., Roberts, M Durrant, J Kirk, B Catovsky D and Beard, M. E. J Blast crisis of chronic myeloid leukemia (CML). II Cell surface marker analysis of 'lymphoid' and myeloid cases Br J Haemat. *34* 179–192 (1976).

Dr Fumimaro Takaku, MD,
Department of Medicine, Jichi Medical School,
3311 1 Yakushiji, Minamikawachi Machi,
Kawachi-gun, Tochigi Prefecture 329-04
(Japan)

Acta haemat. 62 148–152 (1979)

Anion Gap in Multiple Myeloma

Giorgio Paladini and Pietro G. Sala

Divisione di Medicina 1 Istituto di Chimica Clinica, Ospedale Regionale, Udine

Key Words. Anion gap Multiple myeloma Paraproteins

Abstract. The anion gap was evaluated in 26 consecutive patients with multiple myeloma (15 IgG and 11 IgA) The average anion gap was found reduced only in IgG myeloma. Instead in the subjects with IgA myeloma it resulted as increased. The partial differences between our results and those of other authors are discussed. The finding of correlations between the anion gaps and paraproteinemic concentrations (direct in IgA myeloma, inverse in IgG myeloma) gives further support to the conception that the charge exhibited in the serum by IgG and IgA paraproteins is, respectively positive and negative It is suggested that a 'normalization of the anion gap during chemotherapy may be a useful tool to judge the efficacy of the treatment of multiple myeloma.

Introduction

The anion gap is determined by the difference between serum unmeasured anions and unmeasured cations. Its usefulness for the evaluation of metabolic acidosis is well known [3 7 10] A normal anion gap suggests a hyperchloremic acidosis, an increased one indicates a metabolic acidosis sustained by diseases producing retention of anions other than chloride (uremic and lactic acidosis, diabetic and alcoholic ketoacidosis, salicylate, methanol paraldehyde, ethylene glycol poisoning)

Less known are the pathologic conditions associated with a low anion gap. In the clinical practice severe hypoalbuminemia is perhaps the commonest cause of a decreased anion gap [7] Moreover a reduction of the anion gap has been shown also in multiple myeloma [4–6 11] In hypoalbuminemia the reduced anion gap derives from a lowered serum concentration of negatively charged molecules, whereas in multiple myeloma it seemed to be directly affected by the accumulation in the serum of cationic paraproteins [6] Nevertheless, recently *De Troyer et al* [2] were able to confirm the reduction of the anion gap only in IgG myeloma. According to these authors, IgA paraproteins, differently from IgG have isoelectric points slightly below

physiologic pH, therefore, they behave like anions in the serum. However in spite of that finding, the anion gaps in IgA myeloma were normal and not high as expected. On the other hand, *Schnur et al* [9] even confirming the decrease of the anion gap in a group of subjects with asymptomatic plasma cell dyscrasias, could not find any evident correlation between the anion gap and the concentration or type of monoclonal immunoglobulin.

The lack of agreement shown in the above-mentioned studies pushed us to review this subject. The results of our investigation on 26 patients with multiple myeloma and normal or slightly decreased albuminemia confirm the reduction of the anion gap in IgG myeloma and demonstrate its rise in IgA myeloma.

Patients and Methods

26 consecutive patients with multiple myeloma and 30 normal controls were studied in the period from 1st October 1977 to 30th September 1978. Subjects with benign monoclonal gammopathy and patients with albuminemia less than 3 g/dl were excluded from our research. The diagnosis of multiple myeloma was assessed according to the criteria proposed by the Committee of the Chronic Leukemia-Myeloma Task Force [1]. Immunoelectrophoresis showed 15 IgG and 11 IgA monoclonal immunoglobulins in the serum. The concentration of serum paraproteins of each type was measured by single radial immunodiffusion. No patient showed hypernatremia ($Na > 142$ mEq/l), hyperkalemia ($K > 5$ mEq/l), hypermagnesemia ($Mg^{++} > 2.00$ mEq/l) or hypercalcemia ($Ca^{++} > 5$ mEq/l). In every one the renal function was normal (blood urea nitrogen < 25 mg%/ and creatinine < 1.5 mg%). No patient took lithium, bromide or other drugs with cationic charge and in no case did we note any associated pathologic conditions whatsoever which might influence the anion gap. This was calculated by the formula $(N + K^+) - (Cl^- + HCO_3^-)$. Serum sodium, potassium, chloride were measured by Technicon S and venous bicarbonate was estimated (8 CO_2 apparatus set (Harleco)).

Student's tests were used for statistical evaluations of differences between the data of the patients and of the controls. The values of the anion gap were compared to the correspondent concentrations of serum monoclonal immunoglobulins and correlation coefficients (r) were calculated.

Results

The average anion gap of the controls and that one of the patients with multiple myeloma are compared in table I. The patients with myeloma showed a mean anion gap slightly lower than that one showed in the controls ($p = 0.05$). Moreover the anion gap evaluation with reference to classes of monoclonal immunoglobulins showed notably different values in the patients with IgG myeloma if compared to those with IgA myeloma. The mean (± SD) anion gap was 10.7 ± 1.54 mEq/l in IgG myeloma and 18.7 ± 2.08 mEq/l in IgA myeloma, such a difference being highly significant

Table I. The anion gap in controls and patients with multiple myeloma

Group		Anion gap, mEq/l
Controls	30	16.1 ± 1.74
Multiple myeloma		
All	26	14.1 ± 4.46*
IgG	15	10.7 ± 1.54++
IgA	11	18.7 ± 2.08

The anion gap values are means ± SD. The significance levels of the differences between the controls and the multiple myeloma groups are shown. * = 0.05 + p < 0.01 ++ p < 0.001 (Student's test).

Acta haemat. 62 148–152 (1979)

Anion Gap in Multiple Myeloma

Giorgio Paladini and Pietro G Sala

Divisione di Medicina 1 Istituto di Chimica Clinica, Ospedale Regionale, Udine

Key Words. Anion gap Multiple myeloma Paraproteins

Abstract. The anion gap was evaluated in 26 consecutive patients with multiple myeloma (15 IgG and 11 IgA) The average anion gap was found reduced only in IgG myeloma. Instead in the subjects with IgA myeloma it resulted as increased. The partial differences between our results and those of other authors are discussed. The finding of correlations between the anion gaps and paraproteinemic concentrations (direct in IgA myeloma, inverse in IgG myeloma) gives further support to the conception that the charge exhibited in the serum by IgG and IgA paraproteins is, respectively positive and negative It is suggested that a normalization of the anion gap during chemotherapy may be a useful tool to judge the efficacy of the treatment of multiple myeloma.

Introduction

The anion gap is determined by the difference between serum unmeasured anions and unmeasured cations. Its usefulness for the evaluation of metabolic acidosis is well known [3 7 10] A normal anion gap suggests a hyperchloremic acidosis, an increased one indicates a metabolic acidosis sustained by diseases producing retention of anions other than chloride (uremic and lactic acidosis, diabetic and alcoholic ketoacidosis, salicylate, methanol paraldehyde ethylene glycol poisoning)

Less known are the pathologic conditions associated with a low anion gap In the clinical practice severe hypoalbuminemia is perhaps the commonest cause of a decreased anion gap [7] Moreover a reduction of the anion gap has been shown also in multiple myeloma [4–6 11] In hypoalbuminemia the reduced anion gap derives from a lowered serum concentration of negatively charged molecules, whereas in multiple myeloma it seemed to be directly affected by the accumulation in the serum of cationic paraproteins [6] Nevertheless, recently *De Troyer et al* [2] were able to confirm the reduction of the anion gap only in IgG myeloma. According to these authors, IgA paraproteins, differently from IgG have isoelectric points slightly below

When the anion gap values of the subjects with IgA myeloma were expressed as a function of the correspondent serum concentrations of monoclonal immunoglobulins, we found a significant correlation ($r = +0.828$ $p < 0.001$) the greater the serum paraprotein concentration, the higher the anion gap (fig. 2) Also in the cases with IgG myeloma a correlation between the anion gaps and the correspondent paraproteinemic concentrations resulted (fig. 2), but with an inverse proportionality ($r = -0.588$ $p < 0.05$).

Discussion

According to some authors [6, 9] the finding of a lowered anion gap, lacking substantial alterations of serum electrolytes (above all Na Mg⁺⁺ Ca⁺⁺) or laboratory mistakes in their measurement, in a subject who has taken no drugs with cationic activity (bromide, lithium, THAM) and who does not show a greatly decreased albuminemia, may indicate the presence of a monoclonal gammopathy (multiple myeloma, asymptomatic monoclonal gammopathy) and then it must induce the usual clinical and laboratory assessment of such affection. In the opinion of the same authors the finding of a normal anion gap in a patient with a monoclonal gammopathy should raise the problem whether a metabolic complication might exist, i.e. a renal acidosis: the retention of unmeasured anions, characteristic of this pathologic condition, could 'normalize' the decreased anion gap of the monoclonal gammopathy Nevertheless, *De Troyer et al.* [2] found that the average anion gap was reduced only in IgG myeloma, whereas in IgA myeloma it was at normal values. the different behavior of the anion gap in the two types of multiple myeloma were explained by the electric charge recorded in the serum by the monoclonal immunoglobulins, cationic for IgG and anionic for IgA. In accordance with such interpretation, the accumulation in the serum of paraproteins with anionic charge should cause an increased anion gap By the above mentioned authors [2] it did not take place in their patients with IgA myeloma possibly because they also presented a reduction of the serum albumin.

In our investigation the mean anion gap of IgG myeloma was lower than that one recorded in the controls, whereas in IgA myeloma it appeared significantly higher than in normal subjects. However we must point out that in our patients with IgA myeloma, differently from those of *De Troyer et al* [2] the rate of albuminemia was normal or only slightly decreased. Moreover in these subjects a direct connection appeared between the various anion gaps and the serum concentrations of IgA immunoglobulins, this being most likely due to their anionic charge. In our patients with IgG myeloma, instead, a significantly inverse relationship appeared between the anion gaps and the serum immunoglobulin concentrations: an increasing serum paraprotein amount was associated with a decreasing anion gap, most likely depending on the contribution of unmeasured cations by the IgG serum paraproteins.

Also in our investigation the mean anion gap was reduced, although slightly if it was estimated by putting together the two myeloma groups. However that does not allow us to state as it is done by other authors [2, 9] that the anion gap is lowered as a whole in multiple myeloma. Such an assumption

(p < 0 001) A clear statistical difference appeared after comparing the mean anion gap of IgG myeloma with that one of the control group (p < 0 001) Particularly it is noteworthy that all our cases with IgG myeloma had an anion gap lower than 12.6 mEq/l, proving, therefore, no overlap between the groups (fig 1) The average anion gap of the patients with IgA myeloma resulted higher than that of the controls (p < 0.01), although a partial overlapping between the two groups was noted (fig. 1).

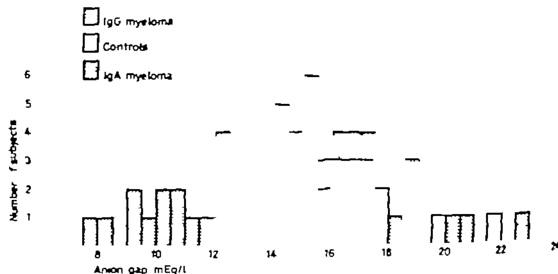


Fig. 1 Distribution of the serum anion gaps in controls and patients with IgG and IgA multiple myeloma

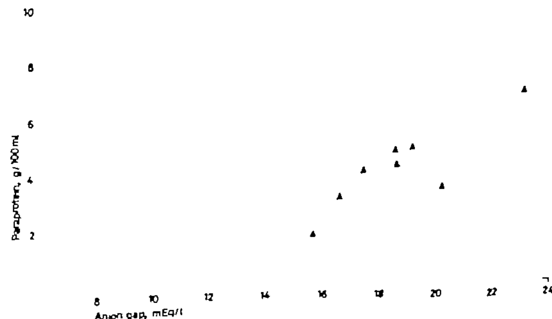


Fig. 2. Relation of the serum anion gaps and paraproteinemic concentrations. ● – IgG myeloma ($r = -0.588$ $p < 0.05$) ▲ – IgA myeloma ($r = +0.828$ $p < 0.001$).

Acta haemat. 62, 153–158 (1979)

HLA Antigens in Idiopathic Thrombocytopenic Purpura

W. A. Veenhoven, J. A. Koars Slijpesteijn and G. S. van der Schans

Division of Haematology, Department of Medicine, University of Groningen, and the Laboratory for Blood Group Serology, University Hospital, Groningen

Key Words. Drug-induced thrombocytopenia · HLA antigens · Idiopathic thrombocytopenic purpura

Abstract. A group of 40 patients with idiopathic thrombocytopenic purpura (ITP) was examined. Typing for HLA A and B antigens was performed. The frequencies of 24 HLA antigens in this group were compared with those of a group of 13 patients with drug-induced thrombocytopenia and two large groups of healthy controls. Differences of statistical significance between these groups were not found. Thus, associations of ITP and particular HLA antigens were not observed. The associations reported by others could not be confirmed.

Introduction

In idiopathic thrombocytopenic purpura (ITP) platelets are removed from the circulation due to accelerated destruction. The destructive mechanism is generally considered to be immunological in nature [2]. Most evidence is in favor of a humoral mechanism [10]. A crucial observation was the demonstration of a transferable plasma factor [8, 13]. Platelet antibodies have been found in 60–70% of cases by several authors using a variety of serological techniques [3, 9, 10].

Drug-induced thrombocytopenia (DTP) is a condition in which platelet destruction is provoked by ingestion of a drug. A humoral immune mechanism is held responsible for the destruction of platelets [2, 9]. Characteristically the thrombocytopenia is reversible within a few weeks after discontinuation of the offending drug [2]. In some patients the thrombocytopenia may be provoked by the ingestion of a certain drug but it is not rapidly reversible after withdrawal of the drug. The distinction between ITP and DTP, therefore, may not always be clear cut. Some authors consider ITP and DTP to be separate diseases, others prefer to think of ITP, DTP and symptomatic thrombocytopenia (secondary to other diseases) as related entities within a syndrome. *Baldini* [2] proposed the designation ITP-syndrome or immunologic thrombocytopenic purpura, *Stijnen* [14] applied the classic term Werlhof's disease in the same sense.

could be acceptable only if the mean anion gap evaluated should express the average of the anion gaps of an equal number of IgG and IgA myelomas. On the contrary in the previous works [2, 9] as well as in ours, the number of cases with IgA myeloma taken into account was smaller than that one of IgG myeloma. Most likely if the two groups were numerically equal the reduced anion gap of a group (IgG) should balance the increased anion gap of the other one (IgA)

Summing up the results of our study confirm the reduction of the anion gap in IgG myeloma only and prove its rise in IgA myeloma. Such a different behavior of the anion gap in the two types of myeloma is likely to be correlated with the different electric charge positive and negative respectively as shown by IgG and IgA monoclonal immunoglobulins in the serum. In our opinion the evaluation of the anion gap in multiple myeloma may be useful to judge the efficacy of the antiblastic treatment as during chemotherapy the normalization of an anion gap previously decreased (IgG myeloma) or increased (IgA myeloma) may mean a reduction of the monoclonal immunoglobulin synthesis and therefore, of the neoplastic proliferation

Acknowledgements

The authors wish to thank Dr *E Sgolfo* for criticism and encouragement, and Miss *Ott rina Trevisan* and Mrs. *Stenia Tosolini* for the editorial assistance.

References

1 Committee of the Chronic Leukemia-Myeloma Task Force, National Cancer Institute: Proposed guidelines for protocol studies. II. Plasma-cell myeloma. Cancer Chemother Rep. *4* 145–158 (1973).
2 De Troyer A., Stolarczyk, A., Zegers de Beyl, D and Stryckmans, P.. Value of anion gap determination in multiple myeloma. New Engl J Med *296*. 858–860 (1977).
3 Emmet, M and Narins, R. G Clinical use of the anion gap Medicine *56*. 38–54 (1977).
4 Frohlich, J Adam, W Golbey M. J and Bernstein, M Decreased anion gap associated with monoclonal and pseudomonoclonal gammopathy Can. med. Ass. J *114* 231–232 (1976).
5 Mikulski, S. M Anion gap and myeloma. New Engl. J Med. *294* 11 (1976).
6 Murray T., Long, W and Narins, R. G. Multiple myeloma and the anion gap. New Engl. J Med. *292* 574–575 (1975).
7 Oh, M S. and Carroll, H. J The anion gap. New Engl. J Med *297* 814–817 (1977).
8 Peters, J P and Van Slyke D D Quantitative clinical chemistry vol. 2 (Williams & Wilkins, Baltimore 1932).
9 Schnur M J Appel G. B., Karp, G and Osserman, E. P The anion gap in asymptomatic plasma cell dyscrasias. Ann. Intern. Med. *86* 304–305 (1977).
10 The anion gap (E) Lancet. *1* 785–786 (1977).
11 Witte, D L., Rodgers, J L., and Barret, D. A The anion gap its use in quality control Clin Chem. *22* 643–646 (1976).

Received December 11 1978
Accepted March 5 1979

Giorgio Paladini, MD
Divisione di Medicina 1 Ospedale Regionale
I-33100 Udine (Italy)

The frequencies of HLA A and B antigens in the groups of patients and controls are given in table I. The differences in respect to 24 HLA antigens are analyzed statistically by the χ^2 test. The highest χ^2 values calculated by comparison of the patient groups with the ET control group are given in table II. A pc value of 0.05 was considered the borderline of significance. The frequencies of HLA antigens in group 1 and group 2 did not differ significantly from each other ($pc > 0.05$). No significant differences were found between these groups, either separately or together and the ET control group ($pc > 0.05$). Furthermore, by comparison of the patient groups with the smaller regional control group the differences were not significant ($p > 0.05$).

Table I. HLA phenotype frequency

HLA antigens	Patient groups						controls, %	
	1		2		1+2			
	n	%		%		%	ET	Reg.
Total	40		13		53		10,000	166
A1	16	40	4	31	20	38	31.8	25.3
A2	18	45	11	85	29	55	53.6	58.4
A3	11	26	4	31	14	28	28.8	29.5
A9 (W23-W24)	9	23	2	15	11	21	19.8	19.3
A10 (25-26)	4	10	0		4	8	7.3	7.2
A11	4	10	2	15	6	11	10.8	7.2
A19 (29 W30-W33)	9	23	1	8	10	19	19.9	20.5
A28	4	10	0		4	8	9.6	11 4
Blank	5	13	2	15	7	13	18.4	21.2
B5	4	10	4	31	8	15	11.2	14.5
B7	12	30	4	31	16	30	27.6	34.7
B8	12	30	3	23	15	28	24 4	26.5
B12 (W44-W45)	6	15	4	31	10	19	25.0	21.7
B13	3	8	0		3	6	5.3	7.8
B14	4	10	0		4	8	3.8	3 6
B15	5	13	2	15	7	13	16.6	13.3
BW16 (W38-W39)	4	10	2	15	6	11	6.3	9 0
B17	1	3	0		1	2	8.6	3.0
B18	3	8	0		2	4	5.7	7.8
BW21 (W49-W50)	3	8	1	8	4	8	2.6	
BW22	2	5	0		2	4	5.4	4 8
B27	4	10	1	8	5	9	8 6	6 0
BW35	4	10	3	23	7	13	17 7	15.0
BW37	4	10	1	8	5	9		3.0
B40	4	10	0		4	8	18.6	15.7
Blank	5	13	2	15	7	13	12.6	11.6

ET = Eurotransplant Reg. = Regional.

Many diseases were found to be associated with increased frequencies of certain HLA antigens [1, 11]. The association may be close as HLA B27 in ankylosing spondylitis, or less impressive though significantly present as seen in some autoimmune disorders. An increased frequency of HLA B8 has been reported in many autoimmune disorders, e.g. myasthenia gravis [1], Graves disease [1] and systemic lupus erythematosus [4]. Recently *Goebel et al.* [6, 7] reported a markedly increased frequency of HLA B8 and B12 in patients with ITP, but not in patients with DTP.

In the past 3 years the HLA patterns in our patients with ITP and our patients with DTP were studied.

Patients and Methods

Diagnostic Criteria

Thrombocytopenia with platelet counts of 70×10^9/liter or less were found on at least 3 successive days. Platelets were electronically counted in EDTA-anticoagulated venous blood and in capillary blood and checked by phase-contrast microscopy and by examination of a blood smear.

Normal or elevated numbers of megakaryocytes were present in the bone marrow. The spleen was not enlarged. The survival of Cr-labelled autologous or homologous platelets, as measured according to *Aster and Jandl*, modified by *Fontain et al.* [5] was shortened to 6 days or less. Platelet loss by bleeding or platelet consumption by diffuse intravascular coagulation were excluded by appropriate clinical and laboratory examinations.

Patients Groups

Patients were classified into one of two groups. If the thrombocytopenia had been provoked by ingestion of a suspect drug that was first prescribed in the previous 4 weeks the condition was classified as DTP (group 2). When there was no drug history or no apparent relationship between drug ingestion and the onset of thrombocytopenia the condition was called ITP (group 1).

Control Groups

The HLA typing in our groups of patients was compared with that of 10,000 controls from Eurotransplant, and in some cases with a group of HLA-typed normal blood donors, examined in the Laboratory for Blood Group Serology, University Hospital, Groningen.

HLA typing was performed by the standard NIH two-stage microcytotoxicity test for at least 24 specificities.

Lymphocyte cytotoxic antibodies were assessed by the microcytotoxicity test mentioned above and by the sensitive long-incubation method described by *Ting et al.* [16]. Patient sera were tested against a panel of lymphocytes obtained from 28 different donors.

Platelet antibodies were assessed by an indirect immunofluorescence technique as previously described [12].

Statistical analysis was performed by χ^2 analysis using a formula including a correction for continuity [15]. In order to exclude HLA antigen frequency deviations on a basis of chance alone p values obtained were multiplied by the number of antigens tested [24], giving a so-called corrected p value (pc).

Results

According to the criteria outlined above a diagnosis of ITP was made in 40 patients (group 1, mean age 44); 29 of them did not ingest any drug at all. 13 patients were classified as DTP (group 2, mean age 54), only 4 of them showing the typical rapid reversibility of thrombocytopenia on withdrawal of the offending drug. All the patients were of Caucasian origin. There was a majority of female patients: 80% in group 1 and 70% in group 2. A history of familial clustering was given by only 1 of the patients with ITP. Unfortunately, further blood examinations were refused by the relative of this patient.

plained by sampling error due to the heterogeneity of the populations examined, by a disease susceptibility as a direct result of the presence of a particular HLA antigen or by linkage disequilibrium between loci on the chromosome 6 coding for HLA antigens and for disease susceptibility [11]. Most disease associations, including the moderate associations found in some autoimmune diseases, may be explained by the latter possibility. Very strong associations, as between HLA B27 and ankylosing spondylitis, may reflect a direct effect of the HLA antigen [1].

As observed by *Rosenberg and Kidd* [11], many reported associations have not been confirmed by subsequent studies. The disagreement between our findings and that of *Goebel et al.* [6, 7] may be explained by sampling error, regional differences in HLA frequency prevalence or by different diagnostic criteria. In respect to the frequency of platelet antibodies as assessed by different techniques in the serum of patients with ITP our percentage and that of *Goebel et al.* are comparable (55 and 65 respectively).

In conclusion, an association of ITP and a particular HLA A or B antigen is not apparent from the present study. The associations reported by *Goebel et al.* [6, 7] could not be confirmed. Experiences of other centers should be published. Studies on the frequencies of HLA D antigens could be important.

Acknowledgements

The authors are indebted to Prof. *H. O. Nieweg*, Prof. *G. J. P. A. Anders* and Dr. *M. R. Halie* for their helpful suggestions, to Dr. *W. J. Sluiter* for advice in statistical analysis and to Eurotransplant for providing their list of HLA frequencies.

References

1 Bach, F. H. and Van Rood, J. J.: The major histocompatibility complex. New Engl. J. Med. *295:* 927–936 (1976).

2 Baldini, M.: Idiopathic thrombocytopenic purpura. New Engl. J. Med. *274:* 1245–1251, 1301–1306, 1360–1367 (1966).

3 Dixon, R. H. and Rosse, W. F.: Platelet antibodies in autoimmune thrombocytopenia. Br. J. Haemat. *31:* 129–134 (1975).

4 Dostal, C., Ivanhyl, D., Macurova, H., Hana, L., and Strejcek, J.: HLA antigens in systemic lupus erythematosus. Ann. rheum. Dis. *36:* 83–85 (1977).

5 Fontein, D. L., Beekhuis, H., Woldring, M. G.; van Zanten, A. K., and Nieweg, H. O.: A test for the effectiveness of splenectomy in Werlhof's disease based on body surface measurement; in Dynamic studies with isotopes in medicine (Int. Atomic Energy Agency, Vienna 1971).

6 Goebel, K. M., Hahn, E., Gassel, W. D. und Havemann, K.: Lymphocytenantigene bei Autoimmun-Thrombocytopenien. Schweiz. med. Wschr. *106:* 1360–1361 (1976).

7 Goebel, K. M., Hahn, E., and Havemann, K.: HLA matching in autoimmune thrombocytopenic purpura. Br. J. Haemat. *35:* 341–342 (1977).

8 Harrington, W. J., Minnich, V., Hollingsworth, J. W., and Moore, C. W.: Demonstration of thrombocytopenic factor in the blood of patients with thrombocytopenic purpura. J. Lab. clin. Invest. *38:* 1–10 (1951).

9 Karpatkin, S.; Strick, N.; Karpatkin, M. B., and Siskind, G. W.: Cumulative experience in the detection of antiplatelet antibody in 234 patients with idiopathic thrombocytopenic purpura, systemic lupus erythematodes and other clinical disorders. Am. J. Med. *52:* 776–785 (1972).

10 McMillan, R.: The pathogenesis of immune thrombocytopenic purpura. C. Clin. Lab. Sci. *8:* 303–331 (1977).

11 Rosenberg, L. E. and Kidd, K. K.: HLA and disease susceptibility: primer. New Engl. J. Med. *297:* 1060–1062 (1977).

12 Schans, G. E. van der, Veenhoven, W. A., Snijder, J. A. M., and Nieweg, H. O.: The de-

Table II. Statistical analysis of differences in HLA antigen frequency between patients and controls

HLA antigens	Patient groups						
	1		2		1 + 2		controls ET n
	n	χ^2	n	χ^2	n	χ^2	
Total	40		13		53		10,000
A1	16	0.93	4	0.10	20	0.65	3,180
A2	18	0.86	11	3.92	29	0.01	5,360
A3	11	0.01	4	0.01	15	0.02	2,880
B5	4	0.04	4	2.73	8	0.62	1,120
B7	12	0.06	4	0.01	16	0.22	2,760
B8	12	0.49	3	0.13	15	0.32	2,440
B12	6	1.55	4	0.09	10	0.66	2,500
B14	4	2.39	0	0.31	4	1.35	380
B40	4	1.30	0	1.70	4	3.99*	1,860

* $p < 0.05$ $pc > 0.05$ (not significant)

IgG platelet antibodies were found in the serum of 12 out of 22 patients (55%) in group 1 and 7 out of 12 (58%) in group 2. Lymphocytotoxic antibodies were found in 6 and 3 of these patients, respectively. By statistical analysis of the differences with respect to HLA antigens between these rather small seropositive groups and controls no significant differences were found ($p > 0.05$).

Discussion

The association of diseases with specific HLA antigens may be of some practical importance for several reasons. It may provide an argument in differential diagnosis. In some persons the relative risk of development of a particular disease can be calculated. Etiological information may be gained and a mode of inheritance may be demonstrated in family studies.

An association of ITP with HLA B8 and HLA B12 was reported by *Goebel et al.* [6, 7], the first antigen being found in 70%, the latter in 45% of 20 patients. The prevalence of both was reduced to about 7% in DTP. A high frequency of the combination of both antigens was found in the ITP patients within one family.

The pattern of HLA frequencies found in our patients does not confirm an association of ITP and a particular HLA antigen. The prevalence of the HLA B8 antigen was slightly increased in the ITP group but did not differ significantly from the control frequency. The HLA B12 frequency was not increased in the ITP group and the frequencies of HLA B8 and B12 were not reduced in the DTP group. Our group of patients with ITP could not be distinguished from the DTP group by different frequencies of particular HLA antigens.

Associations, when present, may be ex

plained by sampling error due to the heterogeneity of the populations examined, by a disease susceptibility as a direct result of the presence of a particular HLA antigen or by linkage disequilibrium between loci on the chromosome 6 coding for HLA antigens and for disease susceptibility [11] Most disease associations, including the moderate associations found in some autoimmune diseases, may be explained by the latter possibility Very strong associations, as between HLA B27 and ankylosing spondylitis, may reflect a direct effect of the HLA antigen [1]

As observed by *Rosenberg and Kidd* [11] many reported associations have not been confirmed by subsequent studies. The disagreement between our findings and that of *Goebel et al* [6, 7] may be explained by sampling error, regional differences in HLA frequency prevalence or by different diagnostic criteria. In respect to the frequency of platelet antibodies as assessed by different techniques in the serum of patients with ITP our percentage and that of *Goebel et al.* are comparable (55 and 65 respectively).

In conclusion, an association of ITP and a particular HLA A or B antigen is not apparent from the present study The associations reported by *Goebel et al.* [6, 7] could not be confirmed. Experiences of other centers should be published. Studies on the frequencies of HLA-D antigens could be important.

Acknowledgements

The authors are indebted to Prof. *H O Nieweg*, Prof. *G J P A. Anders* and Dr *M R. Halie* for their helpful suggestions, to Dr *W J Sluiter* for advice in statistical analysis and to Eurotransplant for providing their list of HLA frequencies.

References

1 Bach, F H. and Van Rood, J J.: The major histocompatibility complex. New Engl. J Med. 295 927–936 (1976).

2 Baldini, M.. Idiopathic thrombocytopenic purpura. New Engl. J Med. 274 1245–1251 1301–1306, 1360–1367 (1966).

3 Dixon, R. H. and Rosse, W F Platelet antibodies in autoimmune thrombocytopenia. Br J. Haemat. 31 129–134 (1975).

4 Dostal, C., Ivanhyi, D Macurova, H.; Hana, I and Strejcek, J HLA antigens in systemic lupus erythematosus. Ann. rheum. Dis. 36. 83–85 (1977).

5 Fontein, D. L., Beekhuis, H., Woldring, M. G., van Zanten, A. K., and Nieweg, H. O.. A test for the effectiveness of splenectomy in Werlhof's disease based on body surface measurement; in Dynamic studies with isotopes in medicine (Int. Atomic Energy Agency Vienna 1971).

6 Goebel, K. M., Hahn, E., Gassel, W D. und Havemann, K.. Lymphozytenantigene bei Autoimmun-Thrombozytopenien. Schweiz. med. Wschr 106 1360–1361 (1976).

7 Goebel, K. M., Hahn, E., and Havemann, K.: HLA matching in autoimmune thrombocytopenic purpura. Br. J Haemat. 35. 341 342 (1977).

8 Harrington, W J Minnich, V.; Hollingsworth, J W and Moore, C. W Demonstration of thrombocytopenic factor in the blood of patients with thrombocytopenic purpura. J Lab. clin. Invest. 38. 1 10 (1951).

9 Karpatkin, S., Strick, N., Karpatkin, M. B., and Siskind, G. W Cumulative experience in the detection of antiplatelet antibody in 234 patients with idiopathic thrombocytopenic purpura, systemic lupus erythematodes and other clinical disorders. Am. J Med. 52 776–785 (1972).

10 McMillan, R.: The pathogenesis of immune thrombocytopenic purpura. C. r Clin. Lab. Sci. 8. 303–331 (1977).

11 Rosenberg, L. E. and Kidd, K. K.. HLA and disease susceptibility primer. New Engl. J Med. 297 1060–1062 (1977).

12 Schans, G. S. van der Veenhoven, W A., Snijder J A. M., and Nieweg, H. O.: The de-

tection of platelet isoantibodies by membrane immunofluorescence. J Lab clin. Med. *90* 4–10 (1977)

13 Shulman, N R., Marder V J and Weinrach, R. S. Similarities between known antiplatelet antibodies and the factor responsible for thrombocytopenia in idiopathic purpura. Ann. N Y Acad. Sci. *124* 499–542 (1965)

14 Stijnen, P J.. Waarnemingen over de pathogenese van de ziekte van Werlhof thesis (VRB Offsetdrukkerij, Groningen 1973)

15 Swinscow T O V.. The χ^2 tests. Br med. J *II.* 513–514 (1976).

16 Ting, A., Hasegawa, T., Ferrone, S., and Reisfeld, R. A.. Presensitization detected by sensitive crossmatch tests. Transplant. Proc. *5.* 813–817 (1973).

Received. December 11, 1978
Accepted. March 3, 1979

W A. Veenhoven, Division of Haematology
Department of Medicine,
University of Groningen, Oostersingel 59
Groningen, 9713 EZ (The Netherlands)

Acta haemat. 62: 159–166 (1979)

Bacterial Infections and Thrombocytopenia in Chronic Idiopathic Thrombocytopenic Purpura

W. A. Veenhoven, M. R. Halie, P. J. Stijnen and H. O. Nieweg

Division of Hematology, Department of Medicine, University of Groningen, Groningen

Key Words. Bacterial infections · ITP · Thrombocytopenia

Abstract. A bacterial infection was considered to be responsible for provoking or maintaining episodes of thrombocytopenia in 5 patients with chronic idiopathic thrombocytopenic purpura (ITP). In 3 patients the course of the disease was continuous and in 2 of them remission was attained after eradication of the infection. In the other 2 patients the course was intermittent and a number of relapses was provoked by the infection. The possible causal connection of chronic ITP and bacterial infections is briefly discussed.

Introduction

Idiopathic thrombocytopenic purpura (ITP) is characterized by thrombocytopenia due to accelerated destruction of platelets which is believed to be mediated by an immune mechanism. The available evidence is in favor of a humoral mechanism. The subject has been extensively reviewed by *Baldini* [2] and more recently by *McMillan* [7].

The course of the disorder may be either acute or chronic. The acute form is self-limiting: improvement usually occurs within 3 months. A history of a preceding viral infection is obtained in two thirds of the patients, about 80% of which have rubella [10]. The chronic form predominantly occurs in adults, more frequently in females. A history of previous bleeding is often obtained and thrombocytopenia persists for longer than 3 months. The course may be continuous or intermittent [2, 13]. In the latter case it is characterized by remissions and relapses. A relapse may follow a viral infection [2, 13] or rarely tuberculosis [5] or histoplasmosis [1]. In some text books [9, 13, 14] the possibility of a preceding bacterial infection is mentioned but surprisingly few cases have been clearly documented.

In the present paper 5 patients with chronic ITP are described in whom episodes of thrombocytopenia were associated with bacterial infection.

Methods

Platelet counts were performed with an electronic particle counter (Coulter Electronics, model FN) and by phase contrast microscopy. The presence of thrombocytopenia was confirmed by the examination of a blood smear.

tection of platelet isoantibodies by membrane immunofluorescence. J Lab. clin. Med. *90* 4–10 (1977).
13 Shulman, N R. Marder V J., and Weinrach, R. S., Similarities between known antiplatelet antibodies and the factor responsible for thrombocytopenia in idiopathic purpura. Ann. N Y Acad. Sci. *124* 499–542 (1965).
14 Sijnen, P J., Waarnemingen over de pathogenese van de ziekte van Werlhof thesis (VRB Offsetdrukkerij, Groningen 1973).
15 Swinscow T O V., The χ^2 tests. Br med. J *ii*, 513–514 (1976)
16 Ting, A., Hasegawa, T Ferrone, S and Reisfeld, R. A., Presensitization detected by sensitive crossmatch tests. Transplant. Proc. *5* 813–817 (1973).

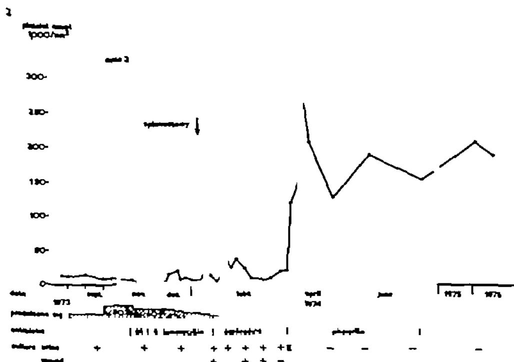
Received. December 11, 1978
Accepted. March 3 1979

W A. Veenhoven, Division of Haematology Department of Medicine,
University of Groningen, Oostersingel 59,
Groningen, 9713 EZ (The Netherlands)

purpura and epistaxis in the past 3 months. There was no history of diarrhea or drug ingestion. Petechiae were observed on the skin. The platelet count was 22,000/µl. A diagnosis of ITP was made. The patient was treated with prednisone and subsequently splenectomy was performed. A remission was not achieved. 2 weeks later diarrhea and abdominal pain developed and Salmonella group B, typhimurium was cultured from the stools. A course of ampicillin was given, the diarrhea disappeared and the platelet count rose to 84,000/µl but it decreased to 8,000 when the antibiotic was stopped after 2 weeks. Ampicillin was reinstituted and remission was attained. Following the discontinuation of treatment with ampicillin the thrombocytopenia recurred twice. On the first occasion, Salmonella was cultured from the stools again. T eatment with prednisone was inef fective. Finally in 1972, a 6-month course of ampicillin was followed by sustained remission. Cultures of the stools remained negative. The course is summarized in figure 1.

Case 2

A man, born in 1906, had long history of easy bruising and frequent epistaxis. Thrombocytopenia with a platelet count of 30,000/µl was found in 1969 In August 1973, ulcerative proctitis, purpura, hypertrophy of the prostatic gland and a urinary tract infection were diagnosed. The platelet count was 6,000/µl. A diagnosis of chronic ITP was made. Treatment with prednisone was started and short course of amoxycillin was given, but in November urinary tract infection recurred. Widespread petechiae were observed. The prostatic gland was enlarged and painful. The platelet count was 2,300/µl. *Escherichia coli* was cultured from the urine. Blood cultures were negative. Treatment with antibiotics was given. The platelet count remained below 10,000/µl. A prostatic abscess was demonstrated by intravenous urography Cultures of the urine performed after prostatic massage, which was given daily remained positive. The dosage of prednisone was gradually reduced. Splenectomy was performed



Platelet survival was measured using ^{51}Cr tagged donor platelets according to *Aster and Jandl* as modified by *Fontein et al.* [4]

Platelet-bound immunoglobulins and complement were detected by a direct immunofluorescence technique using gel-filtered platelets of the patient, as described by *Van der Schans et al.* [11]

Serum platelet antibodies were detected by an indirect immunofluorescence technique using patient sera and donor platelets, as described by *Van der Schans et al.* [12].

Diagnosis

All patients had platelet counts of less than 70,000/μl on repeated occasions. They had normal or increased numbers of megakaryocytes in their bone marrow. Platelet survival was shortened to 24 h or less. The diagnosis of ITP was made by exclusion of other diseases characterized by megakaryocytic thrombocytopenia, as proposed by *Baldini* [2]. Splenomegaly megaloblastic anemia, diffuse intravascular coagulation and bleeding were excluded by appropriate investigations. Antinuclear antibodies were not found. Cultures of the stools, urine, throat and sputum and serological tests for heterophil, antiviral and antitoxoplasma antibodies were negative unless mentioned otherwise. White cell count, granulocyte count and hematocrit were not decreased.

The course of the disease was called chronic when an antecedent bleeding history of 3 months or more was given or when a remission was not attained within 3 months.

Case Records

Case 1

A man, born in 1927 was healthy until 1970 when he was examined because of easy bruising.

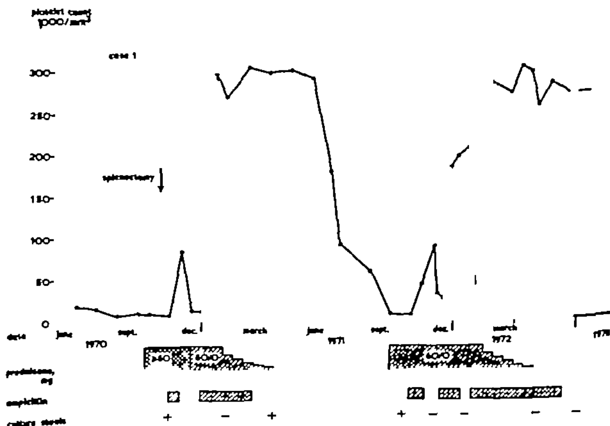


Fig. 1–5. clinical course and platelet counts of cases 1–5. (Fig. 2. ca = Carbenicillin, g = gentamicin; E = epididymitis; prednisone 60 = 60 mg daily 60/0 = 60 mg on alternate days.)

and prednisone but standing remission was not obtained. Splenectomy was performed, but the platelet count rose only transiently. The prednisone dose was increased temporarily and partial remission was achieved. In November 1977 fever, productive cough and 5 days later purpura on the legs once again supervened. In the sputum many granulocytes and few bacteria were found. The platelet count was 8,000/µl. The prednisone dose was temporarily increased and cephradine was given. 5 days later the cough had disappeared and the platelet count was 200,000/µl. Doxycyclin was given prophylactically. A partial remission could be maintained in the next months. The course is shown in figure 4.

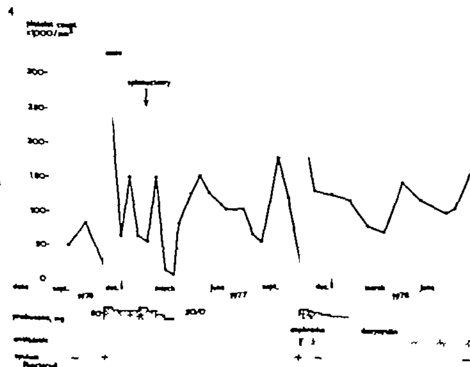
Case 5

A man, born in 1936, was examined in 1976 because of bruising and recurrent epistaxis in the past 3 months. There was a long history of easy bruising, but not of painful micturition or drug ingestion. No petechiae or recent bruises were observed. The prostate gland was enlarged. The platelet count was 22,000/µl. Enterobacter was cultured from the urine, only after prostatic massage. Chronic ITP and bacterial prostatitis were diagnosed. The platelet count decreased to 2,000/ l. Treatment with amoxycillin and prednisone was given and remission was obtained. Cultures of the urine became negative. The prednisone dose was tapered and subsequently stopped. In March 1978, purpura was observed and the platelet count had decreased to 6,000/µl. Citrobacter was cultured from the urine after prostatic massage. Treatment with trimethoprim and sulfamethoxazole was given and the platelet count rose to 120,000/µl. A few weeks later splenectomy was performed and was followed by a sustained remission. The course is shown in figure 5.

Platelet Antibodies

Platelet-bound IgG and IgG platelet antibodies in serum were found in patients 3 and 5 but not in the other patients. Platelet-bound complement

4



and resulted in a transient increase of the platelet count. A surgical wound infection with *Staphylococcus aureus* supervened and was successfully treated with cephradine. 2 weeks later the physical signs of epididymitis became apparent. Cephradine was now replaced by ampicillin. Cultures of the urine that had been positive up to that time now became negative. A few days later the platelet count rose to 290,000/μl and remained within the normal range. The course is shown in figure 2.

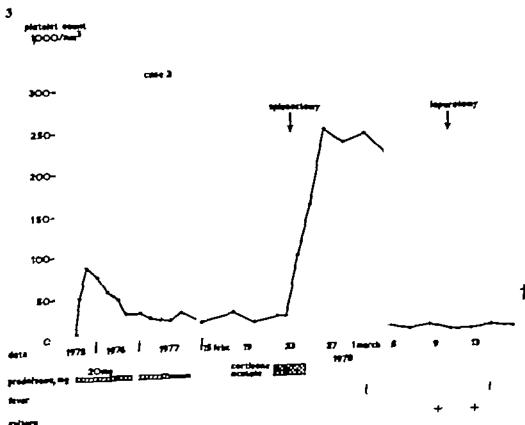
Case 3

A woman, born in 1910, never had bleeding manifestations until the end of 1975 when purpura was observed. The platelet count was 3,000/μl. A diagnosis of ITP was made. She was treated with prednisone 20 mg daily for 1 year but the platelet count did not exceed 40,000/μl. In the next months the prednisone dosage was gradually reduced to zero and the platelet count remained unchanged. Splenectomy was performed and was followed by a prompt remission. 10 days later fever supervened and the platelet count decreased to 8,000/μl. Blood cultures were repeatedly negative. Repeated coagulation studies did not show any signs of diffuse intravascular coagulation. The levels of fibrin degradation products were not increased. 6 days later a second laparotomy was performed. Multiple abdominal abscesses from which *Pseudomonas aeruginosa* was cultured were found. The patient died a few days later. The course is shown in figure 3.

Case 4

A woman, born in 1931, had a long history of recurrent respiratory tract infections with production of purulent sputum. In November 1976, 2 weeks after such an infection, purpura was observed. In the previous 2 years she had noticed the same red spots on her legs following periods of productive cough on two occasions. The platelet count was 44 000/μl. A diagnosis of ITP was made. Many granulocytes, gram-positive cocci and gram-negative rods were found in the sputum and not in the pharynx. Tests for viral antibodies were negative. The patient was treated with ampicillin

3



and prednisone but standing remission was not obtained. Splenectomy was performed, but the platelet count rose only transiently. The prednisone dose was increased temporarily and partial remission was achieved. In November 1977 fever productive cough and 5 days later purpura on the legs once again supervened. In the sputum many granulocytes and few bacteria were found. The platelet count was 8,000/µl. The prednisone dose was temporarily increased and cephradine was given. 5 days later the cough had disappeared and the platelet count was 200,000/µl. Doxycyclin was given prophylactically. A partial remission could be maintained in the next months. The course is shown in figure 4.

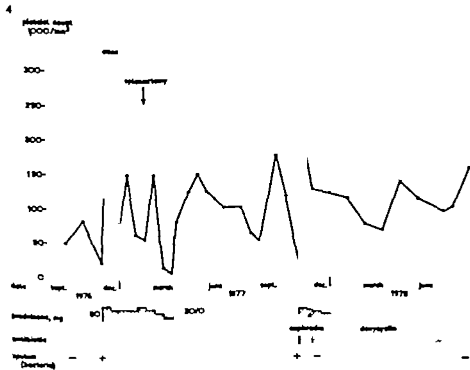
Case 5

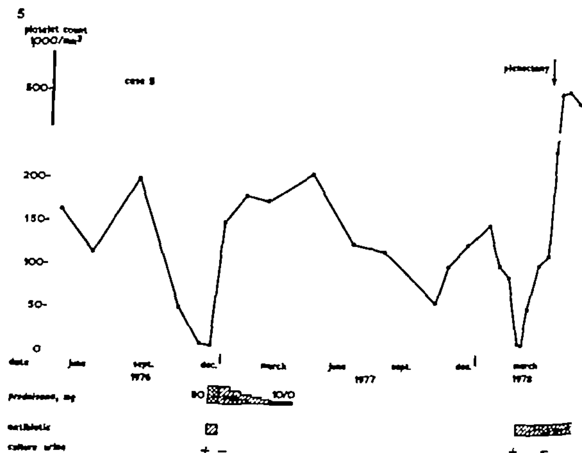
A man, born in 1936, was examined in 1976 because of bruising and recurrent epistaxis in the past 3 months. There was long history of easy bruising, but not of painful micturition or drug ingestion. *No petechiae or recent bruises* were observed. The prostate gland was enlarged. The platelet count was 22,000/µl. Enterobacter was cultured from the urine, only after prostatic massage. Chronic ITP and bacterial prostatitis were diagnosed. The platelet count decreased to 2,000/µl. Treatment with amoxycillin and prednisone was given and remission was obtained. Cultures of the urine became negative. The prednisone dose was tapered and subsequently stopped. In March 1978, purpura was observed and the platelet count had decreased to 6,000/µl. Citrobacter was cultured from the urine after prostatic massage. Treatment with trimethoprim and sulfamethoxazole was given and the platelet count rose to 120,000/µl. A few weeks later splenectomy was performed and was followed by sustained remission. The course is shown in figure 5.

Platelet Antibodies

Platelet-bound IgG and IgG platelet antibodies in serum were found in patients 3 and 5 but not in the other patients. Platelet-bound complement

4





C_3 was not detected. In patient 5 *the antibodies* in serum disappeared when remission was obtained.

Discussion

Thrombocytopenia may occur in the course of infectious diseases by mechanisms *different from immune destruction of* platelets. It is a well known complication of septicemia [3] with or without diffuse intravascular coagulation. It may also occur by pooling of platelets in an enlarged spleen. Relapses of thrombocytopenia in patients with aplastic anemia following bacterial infections have been described [15]

In our patients 1 2 and 3 the spontaneous course of chronic ITP was contin uous. In the patients 4 and 5 it showed an *intermittent* character *In all* patients a sustained remission was not attained following treatment with corticosteroids. Splenectomy was not successful in the first 4 patients. In our 5 patients platelet destruction was dependent upon the presence of a bacterial infection. This was demonstrated in the patients 1 and 2 by the attaining of a remission *only following eradication of a long-standing* infection. In the other 3 patients one or more relapses of thrombocytopenia were provoked by an infection and remissions were obtained following appropriate antibiotic treatment in 2 of them.

Platelet-bound IgG and IgG antibodies in the serum were found in 2 of our 5 patients in the active phase of the disease. Platelet-bound IgG was detected in 8 out of 22 of our own patients with ITP (36%) and

platelet antibodies in 13 out of 22 patients (59%) [to be published]. The frequencies of patients with platelet antibodies in these groups and our present 5 patients do not differ grossly.

The mechanism of platelet destruction following infections is not clear. A nonimmune destruction by loading of platelets with the offending organisms as a mechanism is unlikely because bacteremia has not been found. There are several possible immune-mediated mechanisms. Immune complexes may be formed from microbial antigens and antibodies. They may combine with platelets and shorten their survival [2]. The presence of immune complexes in sera of patients with ITP, most of which were HB_sAg-positive, has been reported [6]. A mechanism like this could only act when viral or bacterial antigens were present in the blood. It should disappear within a few weeks after eradication of the infection. Evidence of immune complexes on our patients' platelets was not obtained, as surface complement was not detected. Another possibility is the action of an antibody with affinity for the platelet surface itself [2]. The surface might have been changed by a nonspecific action of the offending agent. The thrombocytopenia should disappear after treatment of the infection unless cross-reactivity between the microbial antigens and platelet antigens would be present.

Nonspecific activation of the reticulo-endothelial system may be partly responsible for the accelerated platelet destruction. Another additional mechanism in association with an infection may be a decrease in platelet production due to the action of antibodies against megakaryocytes, as was recently suggested by *McMillan et al.* [8].

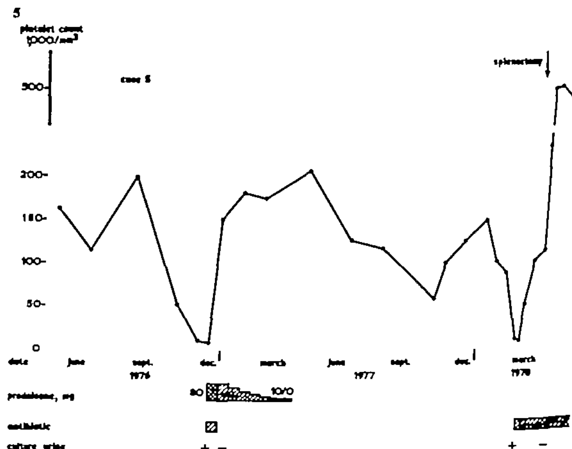
In clinical practice, when chronic ITP has been diagnosed, a vigorous search for infections, including bacterial infections, should be made. It is useful to recall *Allnot*'s [9] statement made in 1944: 'Any possible exciting agents, such as drugs, should be removed.' 'Cure may result following removal of an infectious process.' Its lesson is still valid. Infections should be eradicated before treatment with corticosteroids, splenectomy or vinca alkaloids is instituted.

Acknowledgements

The authors are indebted to *Gerda Thomassen-van der Schans* and *Jennie Hendriks* for skillful technical assistance.

References

1 Armitage, J. O. and Sheets, R. F.: Idiopathic thrombocytopenic purpura in patients with histoplasmosis. J. Am. med. Ass. *237*: 2323–2324 (1977).
2 Baldini, M.: Idiopathic thrombocytopenic purpura. New Engl. J. Med. *274*: 1245–1251, 1301–1306, 1360–1367 (1966).
3 Cohen, P. and Gardner, F. H.: Thrombocytopenia as a laboratory sign and complication of gram-negative bacteremic infection. Archs intern. Med. *117*: 113–124 (1966).
4 Fontein, D. L., Beekhuis, H., Woldring, M. G., van Zanten, A. K., and Nieweg, H. O.: A test for the effectiveness of splenectomy in Werlhof's disease based on body surface measurement; in Dynamic studies with isotopes in medicine (Int. Atomic Energy Agency, Vienna 1971).
5 Kalinowski, S. Z. and Miles Walker, J.: Thrombocytopenic purpura in tuberculosis. Br. J. Tuberc. Dis. Chest *50*: 239–243 (1956).
6 Lurhuma, A. Z.; Riccomi, H., and Masson, P. L.: The occurrence of circulating immune complexes and viral antigens in idiopathic thrombocytopenic purpura. Clin. exp. Immunol. *28*: 49–55 (1977).



C_{3d} was not detected. In patient 5 the antibodies in serum disappeared when remission was obtained.

Discussion

Thrombocytopenia may occur in the course of infectious diseases by mechanisms different from immune destruction of platelets. It is a well known complication of septicemia [3] with or without diffuse intravascular coagulation. It may also occur by pooling of platelets in an enlarged spleen. Relapses of thrombocytopenia in patients with aplastic anemia following bacterial infections have been described [15]

In our patients 1 2 and 3 the spontaneous course of chronic ITP was contin uous. In the patients 4 and 5 it showed an intermittent character. In all patients a sustained remission was not attained following treatment with corticosteroids. Splenectomy was not successful in the first 4 patients. In our 5 patients platelet destruction was dependent upon the presence of a bacterial infection. This was demonstrated in the patients 1 and 2 by the attaining of a remission only following eradication of a long-standing infection. In the other 3 patients one or more relapses of thrombocytopenia were provoked by an infection and remissions were obtained following appropriate antibiotic treatment in 2 of them.

Platelet-bound IgG and IgG antibodies in the serum were found in 2 of our 5 patients in the active phase of the disease. Platelet-bound IgG was detected in 8 out of 22 of our own patients with ITP (36%) and

Acta haemat. 62: 167–172 (1979)

Factor XIII Deficiency

A Family Study by Measurement of Factor XIII Subunits A and S

J. L. Francis and P. J. Todd

Departments of Haematology and Paediatrics, Chester City Hospital, Chester

Key Words. Coagulation disorders · Factor XIII deficiency · Factor XIII subunits

Abstract. A girl with congenital factor XIII deficiency and her large family have been studied by electroimmunoassay of factor XIII subunits A and S. The homozygote has absence of subunit A and a decreased level of subunit S. The heterozygotes have decreased levels of both subunits, and were more readily identified by measurement of subunit A than by the ratio subunit S/subunit A. The mother of the propositus appears to be a new heterozygote, but heterozygosity on the paternal side is traced through three generations.

Introduction

Factor XIII (fibrin-stabilizing factor) is a proenzyme which undergoes activation by thrombin, and in the presence of calcium ions, catalyzes the formation of ε(γ-glutamyl) lysine cross-links between adjacent fibrin monomers [17]. These bonds are formed rapidly between γ-chains of fibrin, but more slowly between α-chains. The β-chains are not involved in cross-linking [18].

Plasma factor XIII is composed of two distinct subunits [19, 20]: subunit A carries the active site and is identical to the fibrin-stabilizing factor in platelets and placenta [5]. Subunit S appears to serve as an extracellular A-chain binding protein [2] and regulates the rate of calcium-dependent activation of the molecule [15]. Some authors use the terms subunit 'a' and 'b' for A and S, respectively, but most work on the immunological characterization of factor XIII deficiency has been performed using the latter terminology.

It has been shown that patients with congenital deficiency of factor XIII lack immunologically identifiable subunit A [6, 12] but have normal [10] or reduced [3, 4, 8] subunit S. In some rare cases subunit S has also been shown to be absent [11].

The condition is considered to be inherited as an autosomal recessive. Previous work suggests that although the two subunits are under separate genetic control, they are genetically related, and heterozygotes may be satisfactorily identified by the ratio subunit S:subunit A [4]. The present study

7 McMillan, R.. The pathogenesis of immune thrombocytopenia. Crit. Rev clin. Lab. Sci. *8* 303–331 (1977)

8 McMillan, R. Luiken, G A., Levy R., Yelenosky R., and Longmire, R. L.. Antibody against megakaryocytes in idiopathic thrombocytopenic purpura. J Am. med. Ass. *239* 2460–2462 (1978)

9 Minot, G R.. Diseases of the blood, in Cecil, A textbook of medicine 6th ed., pp 980–983 (Saunders, Philadelphia 1944)

10 Myllylä, G., Vaheri, A., Vesikari, T., and Penttinen, K. Interaction between human blood platelets, viruses and antibodies. IV Post-rubella thrombocytopenic purpura. Clin. exp Immunol. *4* 323–332 (1969).

11 Schans, G S. Van der Veenhoven, W A., Metting Scherphuis, H. E., and Nieweg, H. O.. Demonstration of platelet membrane associated immunoglobulins and complement in some blood disorders by direct membrane immunofluorescence. Br J Haemat. *35* 679 (1977)

12 Schans, G S. Van der Veenhoven, W A Snijder J A. M., and Nieweg, H. O. The detection of platelet iso-antibodies by membrane immunofluorescence. J clin. Lab Med. *9* 4–10 (1977).

13 Stefanini, M. and Dameshek, W The hemorrhagic disorders, 2nd ed., pp. 159–175 (Grune & Stratton, New York 1962).

14 Williams, W J *et al.* Hematology 2nd ed., p. 1346 (McGraw Hill, New York 1977).

15 Wijnja, L., Koopman, R. K., and Nieweg, H. O.. The influence of infection on the degree of bone marrow insufficiency Acta med. scand. *187* 203–211 (1970).

Received. January 17 1979
Accepted: March 26, 1979

W A. Veenhoven, Division of Haematology
P O Box 30.001, NL–9700 RB Groningen
(The Netherlands)

Acta haemat. 62: 167–172 (1979)

Factor XIII Deficiency

A Family Study by Measurement of Factor XIII Subunits A and S

J. L. Francis and P. J. Todd

Departments of Haematology and Paediatrics, Chester City Hospital, Chester

Key Words. Coagulation disorders · Factor XIII deficiency · Factor XIII subunits

Abstract. A girl with congenital factor XIII deficiency and her large family have been studied by electroimmunoassay of factor XIII subunits A and S. The homozygote has absence of subunit A and a decreased level of subunit S. The heterozygotes have decreased levels of both subunits, and were more readily identified by measurement of subunit A than by the ratio subunit S/subunit A. The mother of the propositus appears to be a new heterozygote, but heterozygosity on the paternal side is traced through three generations.

Introduction

Factor XIII (fibrin-stabilizing factor) is a proenzyme which undergoes activation by thrombin, and in the presence of calcium ions, catalyzes the formation of $\varepsilon(\gamma$-glutamyl) lysine cross-links between adjacent fibrin monomers [17]. These bonds are formed rapidly between γ-chains of fibrin, but more slowly between α-chains. The β-chains are not involved in cross-linking [18].

Plasma factor XIII is composed of two distinct subunits [19, 20]: subunit A carries the active site, and is identical to the fibrin stabilizing factor in platelets and placenta [5]. Subunit S appears to serve as an extracellular A-chain binding protein [ ] and regulates the rate of calcium-dependent activation of the molecule [15]. Some authors use the terms subunit 'a' and 'b' for A and S, respectively, but most work on the immunological characterization of factor XIII deficiency has been performed using the latter terminology.

It has been shown that patients with congenital deficiency of factor XIII lack immunologically identifiable subunit A [6, 12], but have normal [10] or reduced [3, 4, 8] subunit S. In some rare cases subunit S has also been shown to be absent [11].

The condition is considered to be inherited as an autosomal recessive. Previous work suggests that although the two subunits are under separate genetic control, they are genetically related, and heterozygotes may be satisfactorily identified by the ratio subunit S/subunit A [4]. The present study

was performed to determine the concentration of the two factor XIII subunits in plasma from members of the large family of a child with congenital factor XIII deficiency

Materials and Methods

The diagnosis and management of the propositus have been reported previously [9]. Subunits A and S were determined by electroimmunoassay [13 14]. Agarose was dissolved in barbitone buffer pH 8.6, ionic strength 0.03, at a concentration of 1.0%. Anti-A and anti-S (Behringwerke) were added at concentrations of 0.15 and 0.45%, respectively The dimensions of the gels were 185 × 100 × 1.5 mm. Wells of 4 mm diameter were filled with 16 µl of citrated plasma samples. Electrophoresis was carried out at 5 V/cm for 22 h. The plates were washed in 0.2 *M* NaCl overnight, dried and stained with Coomassie blue.

A normal pool was prepared by mixing equal volumes of citrated plasma from 12 normal male and 12 normal female volunteers, and dividing into aliquots. All samples were stored frozen a –20 °C prior to assay and although factor XII biological activity may increase under these condi tions [16] no change in immunoassay values hav been observed [*Francis* unpubl. observations]. I our hands the immunoassay is sensitive to 5% c each protein.

Heterozygosity was defined, in accordanc with previous work [4], by a plasma subunit level less than 2 SD below the mean. Clot solubili ty studies were performed using standard tech niques [1].

Results

The pedigree of the family is shown figure 1. There is no consanguinity and th autosomal recessive inheritance is well illus trated. No precipitation line was noted wi anti-A in the homozygote, and her plas subunit S level was 60%. No other family members were affected clinically or on th basis of clot solubility in 5 *M* urea or 2% acetic acid.

The results of subunits A and S measure ment, and subunit S/subunit A ratios in each family member are presented in table

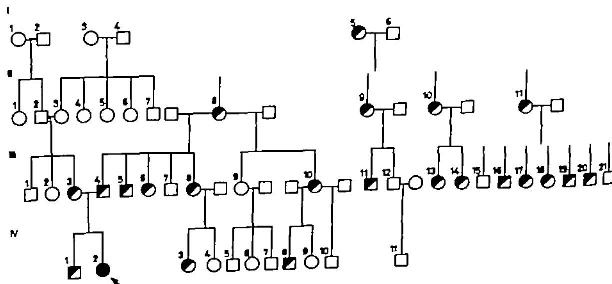


Fig. 1 Pedigree of the family The numbers refer to the immunoassay results as presented in table I. The propositus is arrowed.

Table I. Factor XIII subunits A and S and subunit S/subunit A ratios in family members

		Subunit A %	Subunit S %	Ratio S/A
I	1	deceased		
	2	deceased		
	3	deceased		
	4	deceased	–	
	5	25	80	3.20
	6	deceased		
II	1	105	95	0.90
	2	80	95	1.19
	3	95	95	1.00
	4	95	105	1.10
	5	95	120	1.26
	6	105	105	1.00
	7	95	110	1.16
	8	30	80	2.67
	9	65	110	1.69
	10	25	80	3.20
	11	25	60	2.40
III	1	90	80	0.89
	2	105	95	0.90
	3	45	70	1.55
	4	65	85	1.31
	5	45	75	1.67
	6	40	65	1.62
	7	90	105	1.17
III	8	40	90	2.25
	9	90	85	0.94
	10	60	85	1.42
	11	65	75	1.15
	12	105	105	1.00
	13	45	60	1.33
	14	60	60	1.00
	15	85	80	0.94
	16	65	65	1.00
	17	45	70	1.55
	18	40	70	1.75
	19	50	65	1.30
	20	65	70	1.08
	21	85	90	1.06
IV	1	55	85	1.55
	2	0	60	
	3	50	90	1.80
	4	85	95	1.12
	5	80	90	1.12
	6	95	100	1.05
	7	100	105	1.05
	8	65	70	1.08
	9	90	95	1.06
	10	n. e.	–	–
	11	90	110	1.22

Table II. Statistical analysis of the results in normal controls, normal family members and heterozygotes (mean ± SD)

	Number examined	Subunit A %	Subunit S %	Ratio S/A
Normal controls	24	100.4 12.9	98.5 13.7	0.99 0.14
Normal family members (subunit A > 74%)	20	93.0 8.0	98.0 10.3	1.06 0.11
Heterozygotes (subunit A < 74%)	22	41.6 14.[illegible]	73.4 12.2	1.71 0.65
Homozygote	1	0	60.0	

was performed to determine the concentration of the two factor XIII subunits in plasma from members of the large family of a child with congenital factor XIII deficiency

Materials and Methods

The diagnosis and management of the propositus have been reported previously [9] Subunits A and S were determined by electroimmunoassay [13, 14]. Agarose was dissolved in barbitone buffer pH 8.6, ionic strength 0.03, at a concentration of 1.0%. Anti-A and anti-S (Behringwerke) were added at concentrations of 0.15 and 0.45%, respectively The dimensions of the gels were 185 × 100 × 1.5 mm. Wells of 4 mm diameter were filled with 16 μl of citrated plasma samples. Electrophoresis was carried out at 5 V/cm for 22 h. The plates were washed in 0.2 *M* NaCl overnight, dried and stained with Coomassie blue.

A normal pool was prepared by mixing equal volumes of citrated plasma from 12 normal male and 12 normal female volunteers, and dividing into aliquots. All samples were stored frozen at -20 C prior to assay and although factor XIII biological activity may increase under these conditions [16] no change in immunoassay values have been observed [*Francis* unpubl. observations]. In our hands the immunoassay is sensitive to 5% of each protein.

Heterozygosity was defined, in accordance with previous work [4], by a plasma subunit A level less than 2 SD below the mean. Clot solubility studies were performed using standard techniques [1]

Results

The pedigree of the family is shown in figure 1 There is no consanguinity and the autosomal recessive inheritance is well illustrated. No precipitation line was noted with anti-A in the homozygote, and her plasma subunit S level was 60%. No other family members were affected clinically or on the basis of clot solubility in 5 *M* urea or 2% acetic acid.

The results of subunits A and S measurement, and subunit S/subunit A ratios in each family member are presented in table

Fig. 1. Pedigree of the family The numbers refer to the immunoassay results as presented in table I. The propositus is arrowed.

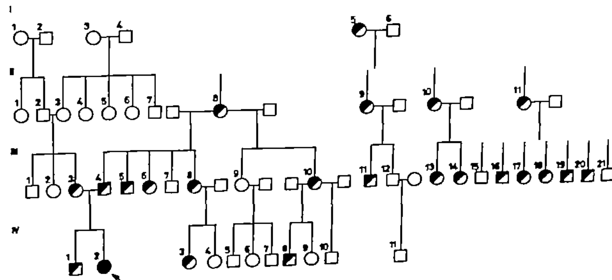


Table I. Factor XIII subunits A and S and subunit S/subunit A ratios in family members

		Subunit A %	Subunit S %	Ratio S/A
I	1	deceased		–
	2	deceased	–	
	3	deceased	–	
	4	deceased	–	
	5	25	80	3.20
	6	deceased	–	–
II	1	105	95	0.90
	2	80	95	1.19
	3	95	95	1.00
	4	95	105	1.10
	5	95	120	1.26
	6	105	105	1.00
	7	95	110	1.16
	8	30	80	2.67
	9	65	110	1.69
	10	25	80	3.20
	11	25	60	2.40
III	1	90	80	0.89
	2	105	95	0.90
	3	45	70	1.55
	4	65	85	1.31
	5	45	75	1.67
	6	40	65	1.62
	7	90	105	1.17
III	8	40	90	2.25
	9	90	85	0.94
	10	60	85	1.42
	11	65	75	1.15
	12	105	105	1.00
	13	45	60	1.33
	14	60	60	1.00
	15	85	80	0.94
	16	65	65	1.00
	17	45	70	1.55
	18	40	70	1.75
	19	50	65	1.30
	20	65	70	1.08
	21	85	90	1.06
IV	1	55	85	1.55
	2	0	60	
	3	50	90	1.80
	4	85	95	1.12
	5	80	90	1.12
	6	95	100	1.05
	7	100	105	1.05
	8	65	70	1.08
	9	90	95	1.06
	10	n. a.	–	
	11	90	110	1.22

Table II. Statistical analysis of the results in normal controls, normal family members and heterozygotes (mean ± SD)

	Number examined	Subunit A %	Subunit S %	Ratio S/A
Normal controls	24	100.4 12.9	98.5 13.7	0.99 0.14
Normal family members (subunit A > 74%)	20	93.0 8.0	98.0 10.3	1.06 0.11
Heterozygotes (subunit A < 74%)	22	48.6 14.2	75.4 12.2	1.71 0.65
Homozygote	1	0	60.0	

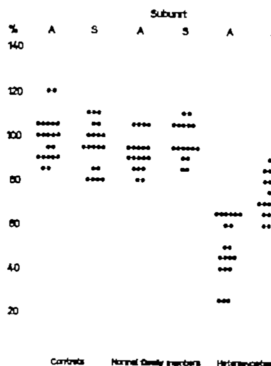


Fig. 2. The distribution of subunits A and S in normal controls, normal family members and heterozygotes.

I. The distribution of subunit A and subunit S levels in normal controls, normal family members and heterozygotes is presented in figure 2. Statistical analysis of the results is shown in table II.

The heterozygotes had levels of subunits A and S significantly lower ($p < 0.001$) than normal controls and normal family members. The ratio subunit S/subunit A was significantly higher ($p < 0.001$) than normal controls and normal family members. Of the 22 family members designated heterozygotes on the basis of their subunit A levels, 17 (77%) had S/A ratios greater than 2 SD above the mean.

Normal subunit S levels were obtained for 2 patients with von Willebrand's disease, excluding cross reaction with factor VIII related antigen as reported by *Girolami et al.* [11]

Discussion

By means of the Laurell electroimmunoassay technique, *Barbui et al.* [4] determined subunit A and S levels in 7 homozygotes and 29 heterozygotes belonging to four families. These workers found a mean subunit S level of 32% and absence of subunit A in homozygotes, and mean levels of 66 and 35%, respectively in heterozygotes. *Girolami et al* [10] reported normal levels of subunit S in their homozygotes, but did not mention the sensitivity of their technique. Later *Girolami et al.* [11] reported cross reaction of their anti-S serum with factor VIII related antigen, and demonstrated that 2 of their patients actually lacked both subunits.

We have reviewed the only available data in heterozygotes studied by immunological techniques [4] and have found considerable variation between families (table III). Subunit A levels are consistently decreased below 65%, but subunit S shows wide variation between individual heterozygotes. We therefore question the conclusion that the subunit S/subunit A ratio is useful in the detection of individual carriers.

Our present work confirms this variation in subunit S levels, and we found the plasma subunit A level more discriminating in the detection of heterozygotes. We have confirmed the results of *Barbui et al* [4] in that our homozygote has absence of subunit A and reduced subunit S, and our heterozygotes have statistically reduced levels of both subunits. In general the higher the plasma subunit A level the less reliable we

Table III. Statistical analysis of the results in the families reported by *Barbui et al.* [4] (mean ± SD)

Family	Number examined	Subunit A %	Subunit S %	Ratio S/A
CER	8	29.4	73.7	2.55
		4.2	14.8	0.58
BREN	7	23.8	72.9	3.07
		2.0	16.0	0.67
NAL	4	53.7	60.0	1.12
		7.5	8.2	0.09
GAL	10	42.0	57.5	1.37
		12.3	15.7	0.19

found the subunit S/subunit A ratio in carrier detection.

The variation in plasma subunit S concentration may be due to genetic linkage of the genes for subunits A and S [4] with variable penetrance of the S gene. This might explain the wide range of S/A ratios that have been observed in heterozygotes for factor XIII deficiency

An alternative hypothesis is that the levels of subunit S are partly governed by the available amount of plasma subunit A by a positive feedback mechanism. This is supported by the finding of *Zimmermann et al.* [21] of a 20% increase in plasma subunit S following administration of factor XIII concentrate (containing subunit A only), to a patient with congenital factor XIII deficiency This finding, however has not been confirmed in other patients [8] or in our own laboratory [*Francis* unpubl. observations].

It was interesting that there were no heterozygotes on the maternal side of the family apart from the mother (III 3) of the propositus. Within the limitations of our definition of heterozygosity we postulate that a new genetic mutation is responsible for our finding, and believe this to be the first report of such an occurrence. The possibility cannot be ruled out, however that there may be some overlap in subunit A levels between the normal and heterozygous populations. This is the largest single family to be studied using sensitive immunological techniques, having been investigated over four generations. No large study has yet been performed to establish the carrier frequency in the general population, but the magnitude of this undertaking has been pointed out by *Duckert* [7]

There remains a need for the re-evaluation of previously reported families with congenital factor XIII deficiency using immunological techniques. This is particularly true for the relatives of those patients reported to have absence of both subunits. Further studies are also required on the synthesis of the factor XIII subunits and the genetical relationship between them.

References

1 Austin, D. E. G. and Rhymes, I. L.: A laboratory manual of blood coagulation; 1st ed., p. 63 (Blackwell, Oxford 1975).

2 Banerjee, D. and Mosesson, M. W.: Characteristics of platelet protransglutaminase (factor XIII) – binding activity in human plasma. Thrombosis and Haemostasis V Congress. Thromb. Diath. haemorrh. 34: 549 (1975).

3 Barbui, T. Cartei, G. Chisesi, T. and Dini, E.: Electroimmunoassay of plasma subunits A and S in case of congenital fibrin stabilizing factor deficiency. Thromb. Diath. haemorrh. 32: 124–131 (1974).

4 Barbui, T. Rodeghiero, F. Dini, E.; Mariani, G. Papa, M. L., Deblasi, R., Cordero Murillo, R., and Montero Umana, C.: Subunits A and S inheritance in four families with congenital factor XIII deficiency. Br. J. Haemat. 38: 267–271 (1978).

5 Bohn, H., Haupt, M. and Kranz, T. M.: Die molekulare Struktur der fibrinstabilisierenden Faktoren des Menschen. Blut 25: 235–248 (1972).

6 Bohn, H., Becker W und Trobrisch, M.. Die molekulare Struktur der fibrinstabilisierenden Faktoren des Menschen. II. Vergleichende immunologische Untersuchungen von Faktor XIII Mangel Plasma und Normalplasma. Blut 6 303–311 (1973).
7 Duckert, F.. Documentation of the plasma factor XIII deficiency in man. Ann. N Y Acad. Sci. 202, 190–199 (1972).
8 Egbring, R., Andrassey K., Havemann, K., Fuchs, G., Ruf, B Schander K., and Trobrisch, H. Experience with long term treatment of congenital factor XIII deficiency with factor XIII concentrate. Blut 33 367–376 (1976)
9 Francis, J and Todd, P.. A case of congenital factor XIII deficiency diagnosed in a neonate and its management. Br med. J ii. 1532 (1978)
10 Girolami, A. Burul, A., and Sticchi, A.. Congenital deficiency of factor XIII with normal subunit S and lack of subunit A. Acta haemat. 58 17–26 (1977).
11 Girolami, A. Burul, A. Fabris, F and Betterle, C.. A tentative classification of factor XIII deficiency in two groups. Acta haemat. 58 318–320 (1977).
12 Israels, E. D., Paraskevas, F and Israels, G L.. Immunological studies of coagulation factor XIII J clin. Invest. 52. 2398–2403 (1973)
13 Laurell, C. B. Quantitative estimation of proteins by electrophoresis in agarose gel containing antibodies. Analyt. Biochem. 15 45–52 (1966).
14 Laurell, C. B Electroimmunoassay Scand. J clin. Lab. Invest. 29 suppl. 124 pp 21–37 (1972).
15 Lorand, L. Gray A. J Brown, K., Credo, R. B., Curtis, C. G Domanik, R. A., and Stenberg, P.. Dissociation of the subunit structure of fibrin stabilizing factor during activation of the zymogen. Biochem. biophys. Res. Commun. 56 914–922 (1974).
16 Losowsky M. S. and Hall, R.. Estimation of plasma fibrin stabilizing factor in families showing congenital deficiencies. Clin. Sci. 30: 171–178 (1966).
17 Pisano, J J Finlayson, J S., and Peyton, M. P.. Cross-link in fibrin polymerised by factor XIII ε(γ-glutamyl) lysine. Science, N.Y 160 892–896 (1968).
18 Pisano, J J., Bronzert, T J., Peyton, M. P and Finlayson, J S.. ε(γ-glutamyl) lysine crosslinks: determination in fibrin from normal and factor XIII-deficient individuals. Ann. N Y Acad. Sci. 202 98–106 (1972).
19 Schwartz, M L., Pizzo, S. V., Mill, R. L., and McKee, P A.. The subunit structures of human plasma and platelet factor XIII (fibrin stabilizing factor). J biol. Chem. 246. 5851–5854 (1971).
20 Schwartz, M. L. Pizzo, S. V., Mill, R. L., and McKee, P A.. Human factor XIII from plasma and platelets; molecular weights, subunit structures, proteolytic activation and crosslinking of fibrinogen and fibrin. J biol. Chem. 248 1395–1407 (1973)
21 Zimmermann, R., Ehlers, W., Mantz, F., Mehrenkan, W., Egbring, R. und Gemmeke, H.. Ein neuer Fall von schwerem Faktor XIII Mangel. Untersuchungen zum Verhalten der Untereinheiten und des Turnover des fibrinstabilisierenden Faktors. Blut 35 457–464 (1977).

Received. December 12, 1978
Accepted. March 20, 1979

J L. Francis, FIMLS,
Department of Haematology
Chester City Hospital, Hoole Lane,
Chester CH2 3EH (England)

Short Communications

Acta haemat. 62: 173–175 (1979)

Genetic Propensity to Benignity in Monoclonal-Gammopathy

Pierre Youinou

Laboratoire du Dr J. Y. Muller, Centre national de Transfusion Sanguine, Paris

Key Words. HLA · Monoclonal gammopathy

Abstract. 52 patients with benign monoclonal gammopathy were HLA typed and compared with 48 patients with malignant monoclonal gammopathy. Antigen B40 was more frequent within the first sample. This association is discussed, together with the results of familial investigations. For systematic serum electrophoresis detected 5 benign monoclonal gammopathies in 172 relatives of 37 patients *suffering from multiple myeloma* or Waldenström's macroglobulinemia.

Introduction

There is a clear connection [7] between benign monoclonal gammopathy (BMG) and multiple myeloma (MM) or Waldenström's macroglobulinemia (WM). Moreover, it is quite difficult to establish benignity without following up the evolution [2]. This is a peculiar question: why do some cases remain asymptomatic throughout several years?

Material and Methods

The frequency of 27 HLA antigens (A1, A2, A3, A9, A10, A11, A28, A29, AW32, AW19, AW19.2; B5, B7, B8, B12, B13, B14, B15, B17, B18, BW21, BW22, B27, BW35, BW37, BW38, BW40) was determined in 52 unrelated BMG patients (follow-up information was obtained at least during 12 months to confirm benignity). Tissue typing was performed using the microlymphocytotoxicity test [1]. The results were compared with those of 48 patients with unrelated malignant gammopathy (including 33 MM and 15 WM) and those of 200 age-matched controls. p values were multiplied by the number of antigens tested (PC) to obtain a more accurate measure of significance [6].

Otherwise, serum electrophoresis was performed in 172 subjects, over 30, relatives of 37 probands (24 MM, 7 WM, 6 BMG). Afterwards, 4 informative families, including one or more monoclonal *gammopathy (MG)* other than the proband's, were investigated (174 subjects).

Results and Discussion

The frequency of B15 antigen (table I) is clearly higher in BMG patients than in

Table I. Frequency of HLA B antigens

Specificity	Controls n 200	MM + WM n = 48	BMG n 52
B5	0.08	0.04	0.15
7	0.29	0.44	0.33
8	0.22	0.31	0.15
12	0.39	0.31	0.19
13	0.02	0	0
14	0.09	0.10	0.04
15	0.05	0.08	0.25
17	0.09	0.06	0.11
18	0.04	0.04	0.08
21	0.05	0.04	0.04
22	0.01	0.02	0.04
27	0.12	0.08	0.08
35	0.12	0.10	0.11
37	0.01	0.10	0
38	0.01	0.02	0
40	0.14	0.02	0.25

* $p < 10^{-4}$ pc < 0.003 (c vs. a) $p < 10^{-4}$ pc < 0.03 (c vs. b).

MM = Multiple myeloma WM = Waldenström's macroglobulinemia BMG = benign monoclonal gammopathy

Table II. Familial data

Age	Monoclonal gammopathy	Diagnosis
Dil. 62*	IgG k	MM
Dil. 55	IgG k	BMG
Gue. 64	IgA l	MM
Gue. 53	IgG l	BMG
Mon. 72*	IgG k	MM
Mon. 83	IgG k	BMG
Min. 61	IgM k	WM
Min. 68	IgM k	WM
Min. 65	IgM k	BMG
Min. 64	IgM k	WM
Min. 62	IgM k	BMG

Proband.

controls ($p < 10^{-4}$ pc < 0.003 relative risk = 6.3) as the rise in BW40 antigen is significant in BMG patients matched by the malignant MG ones ($p < 10^{-4}$ pc < 0.03 relative risk = 16.3) Thus, a MG has 16.3 more chances of being benign when BW40 marker is present in the patient.

The results of familial investigations are summarized in table II. Asymptomatic MG was detected in 4 families 5 BMG versus 3 WM in a single family

The above observations raise some questions First, do B15 subjects and patient's relatives respond to antigen stimulation by increased monoclonal B-cell proliferation? Therefore, is this a familial preneoplastic state, as assumed in some studies [3 5] Then do subjects with B40 (in unselected population and in proband's pedigrees), undergoing an initial stimulus, control better than others this subclone against *second-hit* phenomena [4]?

Unfortunately HLA typing is lacking in these informative families Such analysis would give a better idea of the situation and allow examination of the entire haplotypes.

References

1 Mittal K. K. Mickey M. E. Singal, D P and Terasaki, P I Refinement of the microdroplet lymphocyte cytotoxicity test. Transplantation *6.* 913–927 (1968)

2 Norgaard, O Three cases of multiple myelomas in which the preclinical asymptomatic phases persisted throughout 15 to 24 years. Br J Cancer *25* 417–421 (1971).

3 Odeberg, M. Johansson, B. G and Berlin, S. O., Immunoglobulin analysis in families of myeloma patients. Act. med. scand. *196.* 361–367 (1974).

4 Salmon, S. E. and Seligmann, M.. B-cell neoplasia in man. Lancet *II.* 1230–1233 (1974).

5 Seligmann, M., Danon, F., Mihaesco, C., and Fudenberg, H. H.: Immunoglobulin abnormalities in families of patients with Waldenström's macroglobulinemia. Am. J. Med. *43*: 66–83 (1967).

6 Svejgaard, A., Jersild, C., Staub-Nielsen, L., and Bodmer, W. F.: HL-A and disease-statistical and genetical considerations. Tissue Antigens *4*: 95–105 (1974).

7 Waldenström, J.: The occurrence of benign, essential monoclonal (M-type), non-macromolecular hyperglobulinaemia and its differential diagnosis. Acta med. scand. *176*: 345–365 (1964).

Received: December 14, 1978
Accepted: March 5, 1979

Pierre Youinou, MD
Laboratoire du Dr J. Y. Muller
Centre National de Transfusion Sanguine,
6, rue Alexandre Cabanel, F-75739
Paris Cédex 15 (France)

Table I. Frequency of HLA B antigens

Specificity	Controls n 200	MM + WM n = 48	BMG n 52
B5	0.08	0 04	0.15
7	0.29	0.44	0.33
8	0.22	0.31	0 15
12	0.39	0.31	0 19
13	0.02	0	0
14	0.09	0.10	0.04
15	0.05	0.08	0.25
17	0.09	0.06	0.11
18	0.04	0 04	0.08
21	0.05	0.04	0 04
22	0.01	0.02	0.04
27	0.12	0.08	0.08
35	0.12	0.10	0.11
37	0.01	0 10	0
38	0.01	0.02	0
40	0.14	0.02	0.25

** $p < 10^{-4}$ pc < 0.003 (c vs. a) $p < 10^{-4}$ pc < 0.03 (c vs. b).

MM = Multiple myeloma WM = Waldenström's macroglobulinemia BMG = benign monoclonal gammopathy

Table II. Familial data

Age	Monoclonal gammopathy	Diagnosis
Dil. 62*	IgG k	MM
Dil. 55	IgG k	BMG
Gue. 64	IgA l	MM
Gue. 53	IgG l	BMG
Mon. 72*	IgG k	MM
Mon. 83	IgG k	BMG
Min. 61	IgM k	WM
Min. 68	IgM k	WM
Min. 65	IgM k	BMG
Min. 64	IgM k	WM
Min. 62	IgM k	BMG

* Proband.

controls ($p < 10^{-4}$ pc < 0.003 relative risk = 6.3) as the rise in BW40 antigen is significant in BMG patients matched by the malignant MG ones ($p < 10^{-4}$ pc < 0.03 relative risk = 16.3) Thus, a MG has 16.3 more chances of being benign when BW40 marker is present in the patient.

The results of familial investigations are summarized in table II Asymptomatic MG was detected in 4 families 5 BMG versus 3 WM in a single family

The above observations raise some questions First, do B15 subjects and patient's relatives respond to antigen stimulation by increased monoclonal B-cell proliferation? Therefore is this a familial preneoplastic state, as assumed in some studies [3 5] Then, do subjects with B40 (in unselected population and in proband's pedigrees) undergoing an initial stimulus, control better than others this subclone against second-hit phenomena [4]?

Unfortunately HLA typing is lacking in these informative families. Such analysis would give a better idea of the situation and allow examination of the entire haplotypes.

References

1 Mittal, K. K., Mickey M E. Singal, D P and Terasaki, P I. Refinement of the microdroplet lymphocyte cytotoxicity test. Transplantation 6. 913–927 (1968).

2 Norgaard, O Three cases of multiple myeloma in which the preclinical asymptomatic phases persisted throughout 15 to 24 years. Br J Cancer 25 417–421 (1971).

3 Odeberg, M. Johansson, B G and Berlin, S. O., Immunoglobulin analysis in families of myeloma patients. Act. med. scand. 196. 361–367 (1974)

4 Salmon, S. E. and Seligmann, M B-cell neoplasia in man. Lancet II. 1230–1233 (1974).

Table I. Characteristics of the 16 patients with axillary localizations of HD

Patient No.	Histol. type	Clinical stage	Age	Sex	Professional occupation	Sores of upper limbs	Particularities
1	IV	IA left	53	M	truck driver	?	dead after 74 months survival
2	I	IA left	32	M	worker (painting)	?	dead after 69 months survival
3	II	IA left	25	M	farmer	frequent	–
4	III	IA right	42	M	cook	frequent	
5	III	IA right	44	M	engineer	frequent	built his house with his own hands in the 4 years before diagnosis
6	II	IB left	63	M	worker (packing)	frequent	–
7	III	IA right	42	M	worker (lathe)	frequent	
8	II	IA right	72	M	worker (storing)	frequent	retired for 7 years
9	II	IB left	34	M	worker (masonry)	frequent	–
10	II	IA left	29	M	worker (painting)	frequent	
11	II	IB right	47	M	gardener	frequent	
12	II	IB left	36	M	worker (building)	frequent	
13	II	IA right	29	M	industrial designer	frequent	has bitten his nails (for more than 10 years), frequent infected sores
14	III	IA right	26	F	schoolteacher	frequent	has shaved her arms for 10 years
15	I	IA left	56	F	orker (unskilled)	frequent	
16	II	IA left	24	F	clerk	rare	–

activity could provoke – after a long period of time (10 years or more?) and perhaps in certain genetically sensitive subjects only – a local immune modification (stimulation or impairing?) of the axillary lymph node, which could be an early and necessary step for the action of an infectious agent. It could be either an endogenous agent present in a quiescent state in the body perhaps since birth as suggested by *Vianna et al* [2] or a rare exogenous particle which would have a chance to penetrate only when there is a high rate of hand sores. European work conditions (most workers are men) could be the factor explaining the high proportion of men in this group of axillary localizations.

We do not underestimate the fragility of our hypotheses. However we hope that the data reported here will draw attention on the possible relationship between the initial localization and the etiology of HD

References

1 Levine, P H. An introduction to studies on the etiology of Hodgkin's disease. Cancer Res. 34 1146–1148 (1974).
2 Vianna, N. J.; Greenwald, P and Davies, J N. P.: Nature of Hodgkin disease. Lancet I. 733–736 (1971).
3 Vianna, N. J Evidence for infectious component of Hodgkin disease and related considerations. Cancer Res. 36. 663–666 (1976).

Received: February 19 1979
Accepted. March 20, 1979

J M. Andrieu, Groupe d'Etudes sur la Maladie de Hodgkin, Institut de Recherches sur les Leucémies et les Maladies du Sang, Hôpital Saint-Louis, 2, Place du Dr Fournier F 75010 Paris (France)

Acta haemat. 62 176–177 (1979)

Axillary Hodgkin's Disease in Manual Workers[1]

J M Andrieu H J Weh F Teillet and B Asselain

Groupe d'Etudes sur la Maladie de Hodgkin, Institut de Recherches sur les Leucémies et les Maladies du Sang, Hôpital Saint Louis, Paris

Key Words. Age Axillary localization Hodgkin s disease Manual work Sex

Abstract. Between 1965 and 1974 16 patients were clinically staged as having unique axillary localizations of Hodgkin s disease. Sex ratio (4.3) mean age (40.8 years) and professional occupations (12 out of the 16 patients were engaged in manual work) were significantly different from that of all patients observed during the same period. These facts lead us to suppose the existence of a link between manual work and initial axillary localizations.

Introduction

None of the hypotheses concerning the etiology and infectious nature of Hodgkin s disease (HD) are entirely convincing [1 3] The data reported here, concerning 16 isolated axillary localizations of HD highlight the relationship between initial localization of HD and epidemiological parameters.

Patients, Methods and Results

Between 1965 and 1974 after evaluation including chest X rays, mediastinal tomographies, lymphangiography bone marrow biopsy but no staging laparotomy 16 patients were staged as having unique axillary localizations (clinical stages IA or B). Age, sex and profession were initially noted. The 14 patients alive on the 1 1 1979 answered questions concerning the 3 following points. (1) professional occupation during the 10 years preceding diagnosis, (2) frequency of hand sores, ranged as rare, occasional, frequent; (3) any particularities concerning their upper limbs. Three major points emerge from the results summarized in table I (1) the sex ratio of the 16 patients (4.3 1) is significantly different from that of all patients (1.3 1 $p < 0.005$) observed during the same period (2) their mean age (40.8 years) is much higher than in all patients (28.3 years, $p < 0.01$) (3) 12 out of the 16 patients (75%) had been engaged in manual professional occupation, and 13 out of 14 patients alive on the 1 5-1978 declared having had frequent hand and upper limb sores (precisions in table I) Though the professions of all HD observed in the same period have not yet been recorded, the proportion of manual workers (75%) is no doubt much higher in the axillary form than in all HD On the other hand, this proportion is significantly higher than the percentage of French manual workers (30%) as evaluated by official statistics ($p < 0.001$)

1 This work was partly supported by a grant from AREMAS.

Discussion

These facts lead us to suppose the existence of a link between manual work and initial axillary localizations. Intensive manual

O. N. Ulutin (ed.)
Recent Progress in Blood Coagulation and Thrombosis Research
Bibliotheca Haematologica, No 44.
Karger, Basel 1978
X + 214 p., 70 fig., 33 tab., SFr. 98.
ISBN 3-8055-2896-5

Under the title *Recent Progress in Blood Coagulation and Thrombosis Research*, Ulutin has published papers presented during three different symposia organized as part of the 4th Meeting of the European and African Division of the International Society of Haematology Istanbul, September 5–9, 1977. In the framework of the symposium on vitamin-K-dependent factors various points of interest have been presented: structure and structural analogies between different species, structure-related specificity, use of antibodies for characterization, assay and synthesis *in vitro* of active clotting factors starting with noncarboxylated precursors. The papers presented under the heading 'Fibrinogen and Dysfibrinogenemia' cover a large area from structural considerations to derivatives of fibrinogen in thrombotic states and induced intravascular coagulation. The four papers concerning the antiplatelet drugs review the platelet-vessel interactions, the role of platelets in thromboembolism and the clinical use of antiplatelet drugs. Although there is no claim either of the editor or of the authors of covering the whole field, many of the papers are quite interesting and show some of the various directions of today research in the field of hemostasis. F. Duckert, Basel

J. J. Twomey and *R. A. Good* (eds.)
The Immunopathology of Lymphoreticular Neoplasms
Plenum, New York 1978
XX + 763 pp.; US$ 54.00
ISBN 0-306-33104-7

This book represents volume 4 of the well-introduced series on 'Comprehensive Immunology' which aims at an interdisciplinary treatment of various aspects of experimental and clinical immunology. Accordingly the editors of the present volume resisted the temptation to present yet another proposal for the classification of lymphomas: in fact, the only – and justified – suggestion made is to introduce the term 'primary neoplasms of lymphoid tissues' to replace 'lymphoma', a term that has become too restrictive. The rather voluminous overview is subdivided into 23 chapters, each written by one or a team of specialists in the respective field (with two exceptions all from institutions in the US). The topics covered include competent reviews on the physiology of lymphoid tissues, touching also on developmental and age-related changes, regulatory processes and aspects of cell migration, epidemiology of lymphoreticular neoplasia in animals and man; correlation between immunodeficiency and malignancy of lymphoreticular tissues; various forms of neoplasms of lymphoid tissues; and therapeutic aspects. Access to the content is facilitated by an adequate index, and literature references (in most instances up to 1977) are cited at the end of each chapter. The reviewer shares the editors' hope and anticipation ... that new studies from this point of departure will further improve our classification of these diseases and our understanding of their ... pathogenesis and thereby lead to better approaches toward therapy... This reference book may be highly recommended to pathologists, hematologists, oncologists, clinical immunologists and others involved in the diagnosis and treatment of lymphoreticular neoplasms. M. Hess, Bern

H. J. Day, B. A. Molony, E. E. Nishizawa and *R. H. Rynbrandt*
Thrombosis: Animal and Clinical Models
Advances in Experimental Medicine and Biology, vol. 102
Plenum Press, New York 1978
337 pp. US $ 42.00
ISBN 0-306-40009-X

This book presents the proceedings of a 2-day conference on Animal and Human Models in

Correspondence

Acta haemat. *62* 178 (1979)

Allergic Purpura following Intravenous Administration of Iron Dextran

To the Editor

Severe, even fatal allergic reactions are known to occur following intravenous administration of Imferon (iron dextran USP) [1]

A patient who developed symmetric purpura in the lower extremities following intravenous administration of Imferon is described. To the best of our knowledge, a similar side effect to the above-mentioned drug has not been reported previously

Case Report

A 35-year-old woman was admitted because of severe iron deficiency anemia (hemoglobin 7 g/dl) due to recurrent bleeding from a duodenal ulcer Since she could not tolerate enteral iron preparations and refused intramuscular injections, 1.5 g of Imferon diluted in 1,000 ml of 5% dextrose were administered intravenously There were no immediate side effects to this procedure. However 24 h later for the first time in her life, symmetric purpura appeared on the buttocks, thighs and legs. The platelet count was 274,000 μl, the Rumpel-Leede test was negative and cryoglobulins were not found. The coagulogram was normal. A test for the presence of a migratory inhibitory factor (MIF) to Imferon, performed according to the method of *Kuritzky et al.* [2] was positive. The purpuric rash faded away spontaneously during the following days.

Discussion

Hypersensitivity to parenteral iron preparatio may present with collapse, hypotension, respirato ry distress, cyanosis, facial oedema or urticari [1] The distribution of this patient's rash, simila to that found in Henoch Schoenlein anaphylactoi type of purpura and the positive MIF reaction to Imferon suggest that the purpura was due to hy persensitivity to this drug

References

1 Martindale: The extra pharmacopoeia, 27th ed., pp. 843–844 (1977).

2 Kuritzky A., Livni, E., Munitz, H., Englander T., Tiano, S., Vijsenbeek, H., Joshua, H., and Kott, E. Cell mediated immunity to human basic myelin protein in schizophrenic patients. J neurol. Sci. *30* 369–372 (1976)

Received. February 13, 1979
Accepted March 23, 1979

S. Berliner Y Shoenfeld, E. Livni, J Pinkhas, Department of Internal Medicine 'D' Sackler School of Medicine, Tel Aviv University Beilinson Medical Center Petah Tikva (Israel)

Original Papers

Acta haemat. 62: 181-184 (1979)

Bone Marrow Biopsy in Patients with Malignant Neoplasms Other than Lymphomas or Leukemia

Yoram Cohen, Ruth Gershoni-Baruch and Chaim Lichtic

Northern Israel Oncology Center and Department of Pathology, RAMBAM Medical Center, Faculty of Medicine, Technion, Israel Institute of Technology, Haifa

Key Words. Biopsy · Bone marrow · Malignant neoplasm

Abstract. 104 patients with various cancers, excluding malignant lymphoma and leukemia, underwent bone marrow biopsy using a Jamshidi needle, regular type. In 100 patients an adequate piece of bone marrow was obtained. In 24 patients metastases were detected in the bone marrow. Metastases were found in 10 of 38 (26.3%) patients with breast cancer, in 5 of 17 (29.4%) patients with lung cancer, in 5 of 10 (50%) patients with cancer of the prostate, in 1 patient with rhabdomyosarcoma, 1 with chordoma and in 2 of 14 patients who underwent biopsy in search of unknown cancer. 71% of the patients with positive findings in the bone marrow had clinical signs of bone involvement, 80% had positive X-ray film and 78.9% had positive skeletal isotope survey. Hemogram, serum alkaline phosphatase, serum calcium level and sedimentation rate were of no value in predicting whether the marrow was involved or not. No complications were documented following biopsy. The use of the Jamshidi bone marrow biopsy needle for staging and early detection of metastases in a select group of cancer patients is suggested.

Introduction

There is a general opinion that bone marrow biopsy is superior to bone marrow aspiration in the detection of bone marrow involvement in malignant disorders [4]. In malignant lymphoma the bone marrow biopsy is a routine staging procedure in each new patient [13]. The incidence of metastases in the bone marrow in patients with carcinoma found in autopsies is 34.5% [14]. Various techniques have been developed for bone marrow biopsy: Open surgical biopsy, trephine biopsy [10], the Radner needle [7] and the Craig needle biopsy instrument [5]. In 1971 *Jamshidi and Swain* [12] introduced a new bone marrow biopsy device which enables bone marrow biopsy with unaltered architecture with almost no trauma. The Jamshidi needle is increasingly used in our Oncology Center since 1973 [1, 3]. We

As part of the requirements for an MD thesis at the Sackler Medical School, Tel-Aviv University.

Thrombosis Research held in October 1977 at the Brook Lodge, Augusta, Mich. USA.

The first contribution is a broad review of the significance of platelets and thrombosis in the development of arteriosclerosis. Four contributions then examine various aspects of platelet adhesion, aggregation, secretion of platelet proteins and the effects of blood flow in greater detail The second section consists of seven papers which deal with the complex role of platelets and the endothelial and smooth muscle cells of the blood vessels of rabbits, pigs and primates with experimental intimal hyperplasia. Five papers of the third section 'Clinical Models' focus on platelet-suppressant therapeutic trials in venous and arterial thrombotic conditions such as cardiac thromboembolism, diabetes, cerebral vascular disease, and situations using artificial biomaterials. The last three papers discuss the significance of prostacyclin as well as the relevance of the thrombosis models currently used.

Most authors present few original data but review mainly their own work. The summaries from the transcripts of the discussions after each chapter are very informative seince they point out areas of controversy as well as possible avenues of future research. Most chapters have extensive bibliographies and the book contains, a great help for future look ups, a subject index.

This book is of interest not only to the experimentally working researcher but also to students and clinicians since the covered subjects focus mainly on the current line of research in thrombosis and arteriosclerosis, that is platelet vessel wall interaction. Th. B Tschopp, Basel

Ph. D Ziewe and J Levin
Disorders of Hemostasis.
Major Problems in Internal Medicine, vol. 10
Saunders, Philadelphia, 1976
XVI + 97 pp US$ 12.75
ISBN 0-7216-9685-6

This small booklet is a concise, well-written introduction into the major chapters of the pathophysiology of hemostasis and its clinical disorders. It is obviously designed for students, house officers and internists and hematologists whose major interest is not the field of hemostasis. I have enjoyed the precise, condensed and simple style of the authors. They succeed in describing complicated matters in easily understandable form without sacrificing accuracy The text is supplemented by several excellent color illustrations and by numerous simple and didactic tables and figures.

The text presents generally accepted information and most experts would agree with the majority of the opinions of the authors. Nevertheless, I do believe that most hematologists who have taught hemophiliacs and their families the technique and principles of self-administration of factor VIII or IX preparations would not agree with the authors that 'this approach to the treatment of hemophilia cannot be recommended for general use until more definitive studies are available' Home administration of concentrates has had too much of a positive impact on the morbidity of hemophiliacs to be dealt with in such a cursory statement. Concerning the treatment of hemorrhagic episodes in patients with von Willebrand's syndrome, the authors appear to imply incorrectly that the usually observed rise in F VIII:C level after administration of fresh frozen plasma or of cryoprecipitate well correlates with clinical hemostasis. Many coagulationists would also disagree with the statement that 'there are not enough data to establish the reliability of the activated partial thromboplastin time to monitor the anticoagulant response to heparin therapy' on the other hand, they would agree with the authors, that the whole blood clotting time is an insensitive test to detect deficiencies of the intrinsic system and should be abandoned as a screening test for the detection of hemorrhagic diatheses.

On page 14 a printing error indicates the reference values for circulating fibrin(ogen) degradation products in plasma as < 10 mg/ml instead of μg/ml or mg/L.

Despite these minor criticisms, the book can be wholeheartedly recommended as a valuable and eminently readable summary of the current knowledge of various aspects of normal and defective hemostasis. It should be in every library of a department of medicine.

F Bachmann, MD, Lausanne

Original Papers

Acta haemat. 62: 181-184 (1979)

Bone Marrow Biopsy in Patients with Malignant Neoplasms Other than Lymphomas or Leukemia

Yoram Cohen, Ruth Gershoni-Baruch and Chaim Lichtig

Northern Israel Oncology Center and Department of Pathology, RAMBAM Medical Center, Faculty of Medicine, Technion, Israel Institute of Technology, Haifa

Key Words. Biopsy · Bone marrow · Malignant neoplasm

Abstract. 104 patients with various cancers, excluding malignant lymphoma and leukemia, underwent bone marrow biopsy using a Jamshidi needle, regular type. In 100 patients an adequate piece of bone marrow was obtained. In 24 patients metastases were detected in the bone marrow. Metastases were found in 10 of 38 (26.3%) patients with breast cancer, in 5 of 17 (29.4%) patients with lung cancer, in 5 of 10 (50%) patients with cancer of the prostate, in 1 patient with rhabdomyosarcoma, 1 with chordoma and in 2 of 14 patients who underwent biopsy in search of unknown cancer. 71% of the patients with positive findings in the bone marrow had clinical signs of bone involvement, 50% had positive X-ray film and 78.9% had positive skeletal isotope survey. Hemogram, serum alkaline phosphatase, serum calcium level and sedimentation rate were of no value in predicting whether the marrow was involved or not. No complications were documented following biopsy. The use of the Jamshidi bone marrow biopsy needle for staging and early detection of metastases in a select group of cancer patients is suggested.

Introduction

There is a general opinion that bone marrow biopsy is superior to bone marrow aspiration in the detection of bone marrow involvement in malignant disorders [4]. In malignant lymphoma the bone marrow biopsy is a routine staging procedure in each new patient [13]. The incidence of metastases in the bone marrow in patients with carcinoma found in autopsies is 34.5% [14]. Various techniques have been developed for bone marrow biopsy: Open surgical biopsy, trephine biopsy [10], the Radner needle [7] and the Craig needle biopsy instrument [5]. In 1971 *Jamshidi and Swaim* [12] introduced a new bone marrow biopsy device which enables bone marrow biopsy with unaltered architecture with almost no trauma. The Jamshidi needle is increasingly used in our Oncology Center since 1973 [1, 3]. We

A part of the requirements for an MD thesis at the Sackler Medical School, Tel-Aviv University.

report our data concerning bone marrow biopsy in 100 patients with various cancers, excluding malignant lymphoma and leukemia.

Material and Methods

From July 1975 to September 1976, 104 patients with various malignant tumors excluding malignant lymphoma, underwent 113 bone marrow biopsies, using a Jamshidi needle, regular type. The biopsy was taken in 99 patients from the posterior iliac spine by the technique described by *Jamshidi and Swain* [12]. In 5 patients the biopsy was taken from a suspicious site. No patient was hospitalized for the biopsy which was performed under local anesthesia in the outpatient clinic. The bone marrow piece was fixed in 10% formalin, and decalcified for a few hours. Paraffin sections, 4–6 μm thick, were stained by hematoxylin and eosin dyes and slides were examined by light microscope. Information concerning X-ray bone survey, skeletal isotope scans, sedimentation rate, hemogram, serum calcium and alkaline phosphatase were taken from the patients' charts. Only tests performed 6 weeks prior or after the biopsy were considered.

Results

In 100 patients an adequate piece of bone marrow was obtained. In 4 the biopsies failed because of either too hard or too soft bone yielding little tissue for adequate examination. No complications were documented after the bioptic procedure. There were 42 males and 62 females. The age range was 18–80 years, 2 of the patients were under 30 years and 88 were over 50 years. Table I summarizes the indications for bone marrow biopsy. The biopsies were taken for the following indications: Detection of hematogenous spread to the bone marrow and staging in various malignant

Table I. Indications for bone marrow biopsy and results

Indications	Patients n	Number of positive bone marrow biopsies	Percent of positive bone marrow biopsies
Search for hematogenous spread and staging	73	15	20.5
Obtaining histology	27	9	33.3
Total	100	24	4

Table II. Results of the bone marrow biopsy in the various malignancies

Site of tumor	Patients n	Number of patients with positive bone marrow	Percentage of patients with positive bone marrow
Breast	38	10	26.3
Lung	17	5	29.4
Prostate	10	5	50.0
Others	21	2	9.5
No diagnosis	14		14.–
Total	100	24	24

disorders which are prone to metastases to the bone marrow like oat cell carcinoma of the lung, and in an attempt to obtain diagnosis in patients without histologic confirmation of cancer.

Table II indicates the results of the bone marrow biopsy in the various malignancies. The bone marrow biopsy was positive in 10 out of 38 patients (26.3 /o) with breast cancer, in 5 out of 17 patients (29.4) with lung cancer, in 5 out of 10 patients (50%) with cancer of the prostate

Table III. Correlation of bone marrow results to the clinical and laboratory evaluation of the patients

Type of examination	Patients n	Positive marrow		Negative marrow		
		number of suspected patients	% correlation	number of patients	number of suspected patients	% negative correlation
Clinical evaluation	24	17	70.8	76	27	35.5
X-ray film	20	16	80	59	22	37.3
Isotope scan	19	15	78.9	62	38	61.3
Serum alkaline phosphatase	15	6	40	47	10	21.3
Serum calcium	17	2	11.8	41	0	0

Table III indicates the correlation between the results of bone marrow biopsies and the clinical evaluation of the patients. The bone marrow biopsy was positive in 70.8% of the patients with clinical signs of bone involvement, in 80% of the patients with positive X ray films and in 78.9% of the patients with positive skeletal isotope scan. The serum alkaline phosphatase level was elevated only in 40% of the patients with positive bone marrow and only in 11.8% of the patients the serum calcium level was abnormal. When the bone marrow biopsy was negative there was suspicion of bone metastases in 35.5% on clinical grounds, 37.3% on X ray film basis and 61.3% on skeletal isotope basis. There was no difference in the hemoglobin level, white blood count, thrombocytes count and the sedimentation rate with either positive or negative bone marrow biopsy.

Discussion

In certain malignancies, like breast, lung and prostate cancers, blood borne metastases to the marrow are frequent. Antemortem detection of such metastases can completely alter the course of treatment. Early detection of bone metastases in a patient with breast cancer can save mastectomy and/or aggressive local irradiation. Skeletal isotope scan might be useful in the early detection of bone metastases in patients with cancer of the breast prior to operation [8]. In this respect bone marrow biopsy can be used for pathological staging in locally advanced cases where there is a high likelihood of bone marrow metastases. The same is true in patients with oat cell carcinoma of the lung [11] and cancer of the prostate [6]. In 1956 *Ackerman* [1] suggested bone marrow biopsy as an alternative to bone marrow aspiration. It was found that bone marrow biopsy is superior to bone marrow aspiration in detecting a higher percentage of metastases to the marrow [4, 9]. In bone marrow biopsy the bone architecture is preserved and malignant cells are more readily identified. The aggregation tendency of malignant cells and the fibrotic reaction of the marrow to their presence cause difficulties in obtaining adequate samples of marrow by the aspiration technique. *Contreras et al.* [4], in a survey of 4,000 bone

report our data concerning bone marrow biopsy in 100 patients with various cancers, excluding malignant lymphoma and leukemia.

Material and Methods

From July 1975 to September 1976, 104 patients with various malignant tumors excluding malignant lymphoma, underwent 113 bone marrow biopsies, using a Jamshidi needle, regular type. The biopsy was taken in 99 patients from the posterior iliac spine by the technique described by *Jamshidi and Swain* [12]. In 5 patients the biopsy was taken from a suspicious site. No patient was hospitalized for the biopsy which was performed under local anesthesia in the outpatient clinic. The bone marrow piece was fixed in 10% formalin, and decalcified for a few hours. Paraffin sections, 4–6 μm thick, were stained by hematoxylin and eosin dyes and slides were examined by light microscope. Information concerning X-ray bone survey, skeletal isotope scans, sedimentation rate, hemogram, serum calcium and alkaline phosphatase were taken from the patients charts. Only tests performed 6 weeks prior or after the biopsy were considered.

Results

In 100 patients an adequate piece of bone marrow was obtained. In 4 the biopsies failed because of either too hard or too soft bone yielding little tissue for adequate examination. No complications were documented after the bioptic procedure. There were 42 males and 62 females. The age range was 18–80 years, 2 of the patients were under 30 years and 88 were over 50 years. Table I summarizes the indications for bone marrow biopsy. The biopsies were taken for the following indications: Detection of hematogenous spread to the bone marrow and staging in various malignant disorders which are prone to metastases to the bone marrow like oat cell carcinoma of the lung and in an attempt to obtain diagnosis in patients without histologic confirmation of cancer.

Table II indicates the results of the bone marrow biopsy in the various malignancies. The bone marrow biopsy was positive in 10 out of 38 patients (26.3%) with breast cancer, in 5 out of 17 patients (29.4%) with lung cancer. In 5 out of 10 patients (50%) with cancer of the prostate

Table I. Indications for bone marrow biopsy and results

Indications	Patients n	Number of positive bone marrow biopsies	Percent of positive bone marrow biopsies
Search for hematogenous spread and staging	73	15	20.5
Obtaining histology	7	9	33.3
Total	100	4	4

Table II. Results of the bone marrow biopsy in the various malignancies

Site of tumor	Patients n	Number of patients with positive bone marrow	Percentage of patients with positive bone marrow
Breast	38	10	26.3
Lung	17	5	29.4
Prostate	10	5	50.0
Others	21	2	9.5
No diagnosis	14	2	14.2
Total	100	4	4

Table III. Correlation of bone marrow results to the clinical and laboratory evaluation of the patients

Type of examination	Patients	Positive marrow		Negative marrow		
		number of suspected patients	% correlation	number of patients	number of suspected patients	% negative correlation
Clinical evaluation	24	17	70.8	76	27	35.5
X-ray film	20	16	80	59	22	37.3
Isotope scan	19	15	78.9	62	38	61.3
Serum alkaline phosphatase	15	6	40	47	10	21.3
Serum calcium	17	2	11.8	41	0	0

Table III indicates the correlation between the results of bone marrow biopsies and the clinical evaluation of the patients. The bone marrow biopsy was positive in 70.8% of the patients with clinical signs of bone involvement, in 80% of the patients with positive X ray films and in 78.9% of the patients with positive skeletal isotope scan. The serum alkaline phosphatase level was elevated only in 40% of the patients with positive bone marrow and only in 11.8% of the patients the serum calcium level was abnormal. When the bone marrow biopsy was negative, there was suspicion of bone metastases in 35.5% on clinical grounds, 37.3% on X ray film basis and 61.3% on skeletal isotope basis. There was no difference in the hemoglobin level white blood count, thrombocytes count and the sedimentation rate with either positive or negative bone marrow biopsy

Discussion

In certain malignancies, like breast, lung and prostate cancers, blood borne metastases to the marrow are frequent. Antemortem detection of such metastases can completely alter the course of treatment. Early detection of bone metastases in a patient with breast cancer can save mastectomy and or aggressive local irradiation. Skeletal isotope scan might be useful in the early detection of bone metastases in patients with cancer of the breast prior to operation [8] In this respect bone marrow biopsy can be used for pathological staging in locally advanced cases where there is a high likelihood of bone marrow metastases. The same is true in patients with oat cell carcinoma of the lung [11] and cancer of the prostate [6] In 1956, *Ackerman* [1] suggested bone marrow biopsy as an alternative to bone marrow aspiration. It was found that bone marrow biopsy is superior to bone marrow aspiration in detecting a higher percentage of metastases to the marrow [4 9] In bone marrow biopsy the bone architecture is preserved and malignant cells are more readily identified. The aggregation tendency of malignant cells and the fibrotic reaction of the marrow to their presence cause difficulties in obtaining adequate samples of marrow by the aspiration technique. *Contreras et al* [4], in a survey of 4,000 bone

report our data concerning bone marrow biopsy in 100 patients with various cancers, excluding malignant lymphoma and leukemia.

Material and Methods

From July 1975 to September 1976, 104 patients with various malignant tumors excluding malignant lymphoma, underwent 113 bone marrow biopsies, using a Jamshidi needle, regular type. The biopsy was taken in 99 patients from the posterior iliac spine by the technique described by *Jamshidi and Swain* [12] In 5 patients the biopsy was taken from a suspicious site. No patient was hospitalized for the biopsy which was performed under local anesthesia in the outpatient clinic. The bone marrow piece was fixed in 10% formalin, and decalcified for a few hours. Paraffin sections, 4–6 μm thick, were stained by hematoxylin and eosin dyes and slides were examined by light microscope. Information concerning X-ray bone survey skeletal isotope scans, sedimentation rate, hemogram, serum calcium and alkaline phosphatase were taken from the patients charts. Only tests performed 6 weeks prior or after the biopsy were considered.

Results

In 100 patients an adequate piece of bone marrow was obtained. In 4 the biopsies failed because of either too hard or too soft bone yielding little tissue for adequate examination No complications were documented after the bioptic procedure There were 42 males and 62 females. The age range was 18–80 years, 2 of the patients were under 30 years and 88 were over 50 years. Table I summarizes the indications for bone marrow biopsy The biopsies were taken for the following indications Detection of hematogenous spread to the bone marrow and staging in various malignant disorders which are prone to metastases to the bone marrow like oat cell carcinoma of the lung, and in an attempt to obtain diagnosis in patients without histologic confirmation of cancer

Table I. Indications for bone marrow biopsy and results

Indications	Patients n	Number of positive bone marrow biopsies	Percent of positive bone marrow biopsies
Search for hemato-genous spread and staging	73	15	20.5
Obtaining histology	27	9	33 3
Total	100	24	4

Table II. Results of the bone marrow biopsy in the various malignancies

Site of tumor	Patients n	Number of patients with positive bone marrow	Percentage of patients with positive bone marrow
Breast	38	10	26.3
Lung	17	5	29 4
Prostate	10	5	90.0
Others	21	2	9.5
No diagnosis	14	2	14.2
Total	100	24	24

Table II indicates the results of the bone marrow biopsy in the various malignancies. The bone marrow biopsy was positive in 10 out of 38 patients (26 3%) with breast cancer in 5 out of 17 patients (29 4%) with lung cancer in 5 out of 10 patients (50%) with cancer of the prostate

Acta haemat. 62: 185–190 (1979)

Alterations of the Cell Surface Morphology in Human T-Rosetting Lymphocytes

An Ultrastructural Study

J. Renau-Piqueras and J. Cervera

Instituto de Investigaciones Citológicas de la Caja de Ahorros (Director: Prof. *S. Grisolía*), Valencia

Key Words. E rosettes · Plasma membrane · T-lymphocytes · Transmission electron microscopy

Abstract. A study of E rosette ultrathin sections has been undertaken in an attempt to analyze changes induced during the T-lymphocyte-sheep red blood cell interaction on the rosetted lymphocyte surface architecture.

The formation of nonimmune rosettes implies different types of attachment between lymphocytes and sheep red blood cells which can affect the rosetted lymphocyte surface morphology: (a) Junctions not involving morphological changes which correspond to bands or point-to-point contact sites. (b) Junctions displaying localized morphological changes, which are revealed mainly in the form of microvilli or digitations. (c) Junctions involving broad morphological changes, revealed primarily by the development of various microvilli or other more complex structures. In both cases, the alterations are found only in those areas of the lymphocyte membrane-bound sheep red blood cells. The modifications that affect the rosetted lymphocyte make the validity of the E-rosette test questionable for studies aiming at the analysis of the lymphocyte surface architecture.

Introduction

The ability of human T-lymphocytes to form rosettes with unsensitized sheep red blood cells (SRBC) is considered the technique of choice for the detection and evaluation of human T-lymphocytes [1–4]. This test (E-rosette test) has been used, therefore, as a T-cell marker in different morphological studies of human peripheral blood T-lymphocytes [7–10–1]. Scanning electron microscopy (SEM) studies have shown that the interaction between human T-lymphocytes and SRBC results in a variety of changes in the surface architecture of the two cell types, which are revealed, primarily by the presence of microvilli [8–10]. However, despite their importance in morphological studies of lymphocytes, very few reports have been focused on the ultrastructural analysis of these alterations.

The purposes of the present study were

marrow aspirations and biopsies, came to the conclusion that the biopsy is unquestionably superior to the aspiration for the detection of bone metastases. Skeletal X ray survey is not sensitive enough for the detection of early bone metastases, the isotope bone scan is more efficient in this respect. Although the clinician has to regard a positive bone scan with some precaution as frequently degenerative changes like bony ridges and osteophytes can result in increased uptake of Tc**ETPA.

Although the additive value of the bone marrow biopsy to isotope survey has not yet been exploited our impression is that the combination of clinical signs, X ray film, isotope scan and bone marrow biopsy is highly effective in detecting osseous and bone marrow metastases. The only disadvantage in using the Jamshidi needle is that the biopsy is taken from the posterior iliac spine or the iliac crest only and not from suspicious sites. This problem might be overcome by selecting bone marrow biopsy sites in bone marrow scintigraphy suspicious areas, using the Craig needle under fluoroscopy Nevertheless, the bone marrow biopsy proved to be an important diagnostic and staging procedure for the detection of bone marrow metastases in patients with solid tumors.

References

1 Ackerman, W Vertebral trephine biopsy Ann Surg. *143* 373–378 (1956).

2 Cohen, Y Cohen, H. Carter H and Auslander L. Bone marrow biopsy in cancer patients. Harefuah 90 167–170 (1976).

3 Cohen, Y Vider A., and Ben-Arie, Y Bone marrow biopsy for the staging of patients with malignant lymphoma. Harefuah *94* 273–274 (1978).

4 Contreras, E. Lawrence, D E., and Lee, R. E., Value of the bone marrow biopsy in the diagnosis of metastatic carcinoma. Cancer *29* 778–785 (1972).

5 Craig, F S., Metastatic and primary lesions of bone. Clin. Orthop *73* 33–38 (1970).

6 Crisp, J Random bone marrow biopsy in the staging of carcinoma of the prostate. Br J Urol. *48* 265–267 (1976)

7 Dombernowsky P Worm, A. M., Hainau, B Hansen, H H and Nissen, N. L. The Radner needle – a bone marrow biopsy device. Scand. J Haematol. *14* 270–273 (1974).

8 El-Domeiri, A. and Shroff, S. Role of preoperative bone scan in carcinoma of the breast. Surgery Gynec. Obstet. *142.* 722–724 (1976).

9 Grann, V Pool, J and Mayer K. Comparative study of bone marrow aspiration and biopsy in patients with neoplastic diseases. Cancer *19* 1898–1900 (1966)

10 Helleberg-Rasmussen, I. and Sondergaard-Peterson, H. Bone marrow biopsy with the Bordier trephine. Scand. J Haematol *14* 123–128 (1975)

11 Hirsch, F., Hansen, H. H. Dombernowsky P and Hainau, B Bone marrow examination in the staging of small cell anaplastic carcinoma of the lung with special reference to subtyping. Cancer *39* 2563–2567 (1977).

12 Jamshidi, K. and Swain, W Bone marrow biopsy with unaltered architecture: a new biopsy device. J Lab clin. Med. *77* 335–342 (1971)

13 Johnson, R. E. DeVita, V T., Kim, L. E., Chabner B R., Chretien, P B Berard, C. N and Johnson, S. K. Patterns of involvement with malignant lymphomas and implication for treatment decision-making. Br J Cancer *31* suppl. II, pp 237–241 (1975)

14 Suprun, H. and Rywlin, A. M Metastatic carcinoma in histologic sections of aspirated bone marrow: a comparative autopsy study Sth. med. J *69* 438–439 (1976)

Received March 27 1979
Accepted March 30, 1979

Yoram Cohen, MD Deputy Director
Northern Israel Oncology Center
Rambam Medical Center Haifa (Israel)

Acta haemat. 62 185–190 (1979)

Alterations of the Cell Surface Morphology in Human T Rosetting Lymphocytes

An Ultrastructural Study

J. Renau-Piqueras and J. Cervera

Instituto de Investigaciones Citológicas de la Caja de Ahorros (Director: Prof. *J. Grisolia*), Valencia

Key Words. E-rosettes · Plasma membrane · T lymphocytes · Transmission electron microscopy

Abstract. A study of E-rosette ultrathin sections has been undertaken in an attempt to analyze changes induced during the T-lymphocyte-sheep red blood cell interaction on the rosetted lymphocyte surface architecture.

The formation of nonimmune rosettes implies different types of attachment between lymphocytes and sheep red blood cells which can affect the rosetted lymphocyte surface morphology: (a) Junctions not involving morphological changes which correspond to bands or point-to-point contact sites. (b) Junctions displaying localized morphological changes, which are revealed mainly in the form of microvilli or digitations. (c) Junctions involving broad morphological changes, revealed primarily by the development of various microvilli or other more complex structures. In both cases, the alterations are found only in those areas of the lymphocyte membrane-bound sheep red blood cells. The modifications that affect the rosetted lymphocyte make the validity of the E rosette test questionable for studies aiming at the analysis of the lymphocyte surface architecture.

Introduction

The ability of human T-lymphocytes to form rosettes with unsensitized sheep red blood cells (SRBC) is considered the technique of choice for the detection and evaluation of human T-lymphocytes [1–4]. This test (E-rosette test) has been used, therefore, as a T-cell marker in different morphological studies of human peripheral blood T lymphocytes [7, 10–1]. Scanning electron microscopy (SEM) studies have shown that the interaction between human T lymphocytes and SRBC results in a variety of changes in the surface architecture of the two cell types, which are revealed, primarily by the presence of microvilli [8–10]. However, despite their importance in morphological studies of lymphocytes, very few reports have been focused on the ultrastructural analysis of these alterations.

The purposes of the present study were

to (a) analyze the effects induced during the T lymphocyte-SRBC interaction on the surface topography by transmission electron microscopy (TEM) and (b) evaluate the use of the E rosette test in morphological studies of the lymphocyte surface.

Material and Methods

Lymphocytes. Peripheral blood was drawn from a healthy young donor. Lymphocytes were isolated from the heparinized blood by centrifugation on Ficoll Isopaque and washed three times with Hanks' balanced salt solution (HBSS). They were then resuspended at a concentration of 4×10^6 cells/ml in HBSS with 25% heat-inactivated fetal calf serum (FCS).

Sheep Red Blood Cells. Freshly drawn SRBC were stored in Alsever's solution (1:1) and kept in sterile vials at 4 °C for a maximum period of 2 weeks. They were washed three times in Alsever's solution and resuspended in 0.5% concentration (8×10^7 cells/ml) in HBSS with 25% heat-inactivated FCS.

Test for E rosettes. E rosettes were prepared according to the method described earlier [1, 11, 12]. Equal volumes (0.25 ml each) of lymphocyte and SRBC suspensions were mixed in a small test tube. After incubation for 60 min at 37 °C, the mixture was centrifuged at 200 *g* for 5 min and incubated at 4 °C overnight. The cells were then carefully resuspended and examined by light microscopy (phase contrast microscopy). The proportion of lymphocytes to which more than three SRBC adhered was determined.

Transmission Electron Microscopy. For TEM, tubes were gently agitated and pellets containing E-rosettes were fixed for 60 min at 4 °C with 2% glutaraldehyde in 0.1 *M* cacodylate buffer (pH 7.4), washed in the same buffer for 120 min at 4 °C and postfixed with 1% osmium tetroxide in 0.28 *M* veronal-acetate buffer (pH 7.4) for 45 min at 4 °C. Sucrose was added to the last solutions to maintain osmolarity (410 mosm). The material was dehydrated through a graded series of ethanol and embedded in Epon 812. Ultrathin sections (interference colorsilver) were cut with glass knives on a LKB ultramicrotone and stained with uranyl acetate and lead citrate. At least 100 electron micrographs of E-rosettes were made with a Philips 300 EM at 60 kV.

Results

Rosette Formation. In the samples studied the proportion of E-rosette forming lymphocytes was estimated to be 63.1 ± 8.7% (mean ± SD). The E rosettes, examined by phase contrast microscopy, were usually large with 9.2 ± 1.5 SRBC per lymphocyte. The SRBC appeared to be preferentially distributed on the portion of the cell membrane more distant from the nucleus. The E rosettes observed by TEM were similar to those described with phase contrast microscopy.

Transmission Electron Microscopy. The examination of a high number of E-rosettes enabled us to establish different types of junctions between lymphocytes and SRBC, which we grouped according to the nature of the alterations induced in the plasma membrane of the rosetted lymphocyte.

(a) Junctions not involving morphological changes in the lymphocyte surface. These correspond to bands or point contact sites which involve definite areas of the membrane (fig. 1–3, 9).

(b) Junctions displaying localized morphological changes. These are revealed mainly in the form of microvilli or digitations and usually appear as isolated figures (fig. 4).

(c) Junctions involving broad morphological changes revealed in diverse manners, the most general being due to the presence of a number of microvilli and digitations in zones of greater SRBC density, as illustrated in figures 5, 7 and 8. There is, in addition, another more complicated alteration

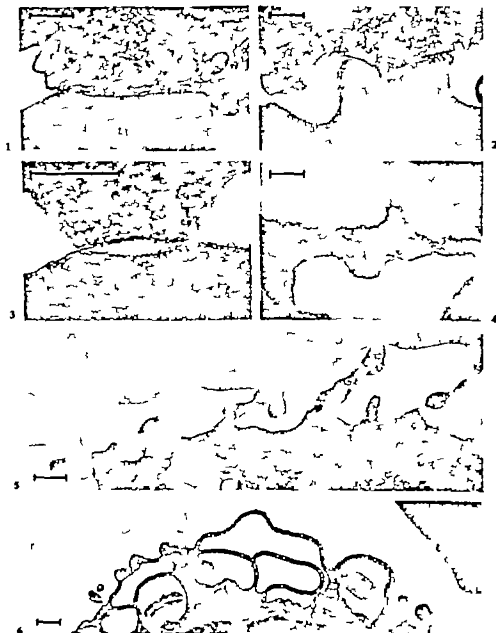


Fig. 1–5. Ultrastructure of different types of binding sites in E-rosettes. (fig. 1, × 61,500; fig. 2, × 47,750; fig. 3, × 119,750; fig. 4, × 45,000; fig. 5, × 4_,000; bar 0 μm).

Fig. 6. Rosetting lymphocyte showing complex and broad alterations in their surface morphology. Bar 0 μm. × 30,750.

to (a) analyze the effects induced during the T lymphocyte SRBC interaction on the sur face topography by transmission electron microscopy (TEM) and (b) evaluate the use of the E rosette test in morphological stud ies of the lymphocyte surface.

Material and Methods

Lymphocytes Peripheral blood was drawn from a healthy young donor Lymphocytes were isolated from the heparinized blood by centrifugation on Ficoll-Isopaque and washed three times with Hanks' balanced salt solution (HBSS) They were then resuspended at a concentration of 4 × 10⁶ cells/ml in HBSS with 25% heat inactivat ed fetal calf serum (FCS)

Sheep Red Blood Cells. Freshly drawn SRBC were stored in Alsever's solution (1 1) and kept in sterile vials at 4 C for a maximum period of 2 weeks. They were washed three times in Alsever's solution and resuspended in 0.5% concentration (8 × 10 cells/ml) in HBSS with 25% heat inactivated FCS.

Test for E-rosettes E-rosettes were prepared according to the method described earlier [1, 11 12] Equal volumes (0.25 ml each) of lymphocyte and SRBC suspensions were mixed in a small test tube. After incubation for 60 min at 37 °C, the mixture was centrifuged at 200 g for 5 min and incubated at 4 C overnight. The cells were then carefully resuspended and examined by light microscopy (phase contrast microscopy). The proportion of lymphocytes to which more than three SRBC adhered was determined.

Transmission Electron Microscopy For TEM, tubes were gently agitated and pellets containing E-rosettes were fixed for 60 min at 4 °C with 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7 4), washed in the same buffer for 120 min at 4 C and postfixed with 1 / osmium tetroxide i 0.28 M veronal-acetate buffer (pH 7 4) for 45 min at 4 °C. Sucrose was added to the last solutions to maintain osmolarity (410 mosm) The material was dehydrated through a graded series of ethanol and embedded in Epon 812. Ultrathin sections (interference color:silver) were cut with glass knives on a LKB ultramicrotome and stained with uranyl acetate and lead citrate. At least 100 electron micrographs of E rosettes were made with a Philips 300 EM at 60 kV

Results

Rosette Formation In the samples studied, the proportion of E-rosette forming lymphocytes was estimated to be 63 1±8.7% (mean ± SD) The E rosettes, examined by phase contrast microscopy were usually large with 9.2 ± 1.5 SRBC per lymphocyte. The SRBC appeared to be preferentially distributed on the portion of the cell membrane more distant from the nucleus. The E rosettes observed by TEM were similar to those described with phase contrast microscopy

Transmission Electron Microscopy The examination of a high number of E-rosettes enabled us to establish different types of junctions between lymphocytes and SRBC, which we grouped according to the nature of the alterations induced in the plasma membrane of the rosetted lymphocyte

(a) Junctions not involving morphological changes in the lymphocyte surface. These correspond to bands or point contact sites which involve definite areas of the membrane (fig. 1–3 9).

(b) Junctions displaying localized morphological changes. These are revealed mainly in the form of microvilli or digitations and usually appear as isolated figures (fig. 4).

(c) Junctions involving broad morphological changes revealed in diverse manners, the most general being due to the presence of a number of microvilli and digitations in zones of greater SRBC density as illustrat ed in figures 5 7 and 8 There is, in addi tion, another more complicated alteration

type (fig. 6). These deformations are found only in lymphocyte membrane areas where there are various numbers of bound SRBC. In zones where SRBC are not present the membrane appears smooth or shows only a few microvilli (fig. 8).

Discussion

It has been demonstrated in different studies performed by SEM that the SRBC lymphocyte interaction in the formation of E-rosettes involves modifications in the surface morphology of the two cell types [8–10]. However due to the resolution and type of image obtained by SEM, the detailed analysis of those modifications and the extent of junction between plasma membranes produced during the lymphocyte SRBC interaction is not possible using this technique.

Our results indicate that, from a morphological point of view the possibilities of attachment between the two cell types involved in E-rosettes are greater than those previously described by SEM and TEM [2, 5 6 8 10] and that many of them involve important changes in the lymphocyte surface topography. Moreover the importance of some cellular structures, such as microvilli, in the formation and maintenance of E-rosettes is suggested. At the same time the fact that microvilli appear almost exclusively in the contact zones with SRBC suggest that microvilli are formed during the process of attachment between T-lymphocytes and SRBC, and that E rosette formation is a dynamic process in which other cellular structures may be involved [3].

Even though quantitative morphological differences between the human T and B peripheral blood lymphocytes have been demonstrated by means of morphometric methods [11 12] one of the most common criteria for morphologically differentiating the T lymphocytes from the B lymphocytes has been the careful study of the topography of the surface of each of these cell types [9 10]. However these criteria have lead to much controversy because differences in the techniques used for SEM and/or in the cellular microenvironments seem to modify the surface characteristics of the lymphocytes [reviewed in references 9 13]. The results presented in this paper together with previous results [8–10], indicate that the modifications that affect the rosetted lymphocytes oblige the cautious use of the rosette formation technique in those studies focused on the characterization of the T and B lymphocytes by an analysis of their surface morphology or in general, in studies dealing with the analysis of the surface topography of lymphocytes.

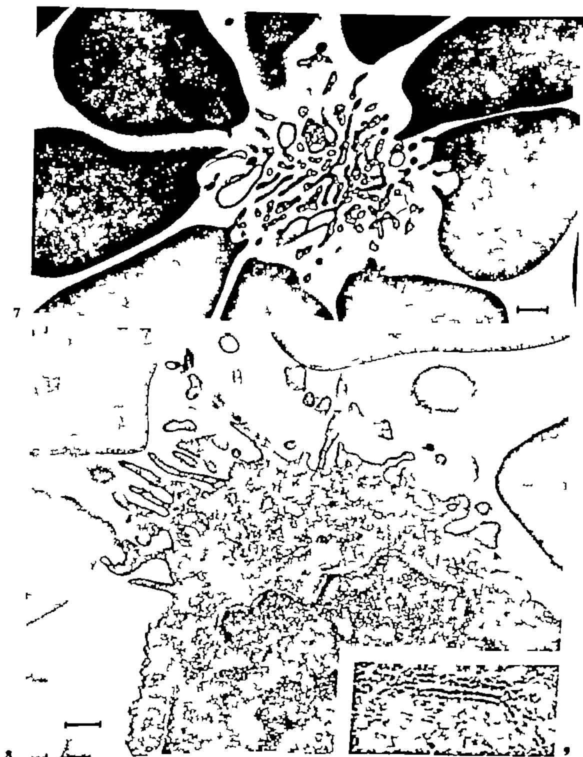
Fig. 7 Micrograph illustrating an E-rosette cross-section and the presence of microvilli in the vicinity of SRBC. Bar 0.5 µm. × 30,500.

Fig. 8. Micrograph showing rosetted lymphocyte and the presence of microvilli in the zone of SRBC attachment. Bar 0.8 µm. × 30,750.

Fig. 9 High magnification of bead type junction. The distance between plasma membranes was constant 40 Å. Bar 0.1 µm. × 46,000.

Acknowledgements

We thank Professor *S. Gramlar* and to Drs. *E. Karcht*, *A. Martinez* and *F. Thompson* for critically reading the manuscript and offering helpful suggestions. We would also like to thank Miss *M. J. Agulló*, Miss *F. Ripoll*, Miss *J. Salazar* and Miss *M. Haly* for their technical assistance.


7
8
9

type (fig. 6). These deformations are found only in lymphocyte membrane areas where there are various numbers of bound SRBC in zones where SRBC are not present the membrane appears smooth or shows only a few microvilli (fig. 8).

Discussion

It has been demonstrated in different studies performed by SEM that the SRBC lymphocyte interaction in the formation of E-rosettes involves modifications in the surface morphology of the two cell types [8–10] However due to the resolution and type of image obtained by SEM the detailed analysis of those modifications and the extent of junction between plasma membranes produced during the lymphocyte SRBC interaction is not possible using this technique

Our results indicate that, from a morphological point of view the possibilities of attachment between the two cell types involved in E-rosettes are greater than those previously described by SEM and TEM [2, 5 6 8 10] and that many of them involve important changes in the lymphocyte surface topography Moreover the importance of some cellular structures, such as microvilli, in the formation and maintenance of E-rosettes is suggested. At the same time, the fact that microvilli appear almost exclusively in the contact zones with SRBC suggest that microvilli are formed during the process of attachment between T-lymphocytes and SRBC, and that E rosette formation is a dynamic process in which other cellular structures may be involved [3]

Even though quantitative morphological differences between the human T and B peripheral blood lymphocytes have been demonstrated by means of morphometric methods [11 12] one of the most common criteria for morphologically differentiating the T lymphocytes from the B lymphocytes has been the careful study of the topography of the surface of each of these cell types [9 10] However these criteria have lead to much controversy because differences in the techniques used for SEM and or in the cellular microenvironments seem to modify the surface characteristics of the lymphocytes [reviewed in references 9 13] The results presented in this paper together with previous results [8–10] indicate that the modifications that affect the rosetted lymphocytes oblige the cautious use of the rosette formation technique in those studies focused on the characterization of the T and B lymphocytes by an analysis of their surface morphology or in general, in studies dealing with the analysis of the surface topography of lymphocytes.

Fig. 7 Micrograph illustrating an E-rosette cross-section and the presence of microvilli in the vicinity of SRBC. Bar 0.5 µm 30,500.

Fig. 8. Micrograph showing rosetted lymphocyte and the presence of microvilli in the zone of SRBC attachment. Bar 0.8 µm 30,790

Fig. 9 High magnification of band type junction. The distance between plasma membranes is constant 40 Å. Bar 0.1 µm. 46,000

Acknowledgements

We thank Professor *S Grisolia* and to Drs *E Knecht A Martinez and F Thompson* for critically reading the manuscript and offering helpful suggestions. We would also like to thank Mrs *M J Agullo* Miss *F Ripoll*, Mrs *I Salazar* and Mrs *M Hale* for their technical assistance

References

1 Bach, J F Evaluation of T-cells and thymic serum factor in man using the rosette technique. Transplant. Rev *16* 196–217 (1973)

2 Bentwich, Z. Douglas, S. D., Siegal, P F and Kunkel, H. G.. Human lymphocyte-sheep erythrocyte rosette formation some characteristics of the interaction. Clin. Immunbiol. Immunopathol. *1* 511–517 (1973).

3 Cohnen, G Fischer K., and Brittinger G.. Human T lymphocyte rosette formation. Inhibition by cytochalasin B. Immunology *29* 337–341 (1972)

4 Jondal, M., Holm, G and Wigzell, H.. Surface markers on human T and B lymphocytes. J exp Med. *136* 207–215 (1972)

5 Kataoka, K. Minowada, J and Pressman, D Electron microscope study on human lymphocyte-sheep erythrocyte rosettes. J natn. Cancer Inst. *55* 1323–1325 (1975)

6 Levy J D Knieser M R., and Briggs, W A.. Ultrastructural characteristics of the non-immune rosette-forming cell. J Cell Sci. *18* 79–96 (1975)

7 Lin, P S Cooper A. G and Worth, H. H Scanning electron microscopy of human T-cell and B-cell rosettes. New Engl J Med. *289* 548–551 (1973).

8 Newell, D G Roath, S., and Smith, J L. The scanning electron microscopy of normal peripheral blood lymphocytes. Br J Haemat. 32 309–316 (1976)

9 Polliack, A.. Normal, transformed and leukemic leukocytes. A scanning electron microscopy atlas (Springer Berlin 1977)

10 Polliack, A., Fu, S M., Douglas, S. D., Bentwich, Z. Lampen, N and De Harven, E. Scanning electron microscopy of human lymphocyte-sheep erythrocyte rosettes. J exp. Med. *140* 146–158 (1974).

11 Renau-Piqueras, J., Cerdan, F Barbera, E, and Cervera, J Electron microscopic morphometric analysis of human T and B peripheral blood lymphocytes. Virchows Arch. Abt. B Zellpath. *28* 47–57 (1978)

12 Renau-Piqueras, J and Knecht, E.. Ultrastructural morphometric characterization of human T and B peripheral blood lypmhocytes. Morfol. Normal Patol. Secc A. *2* 455–463 (1978).

13 Roath, S., Newell, D., Polliack, A., Alexander, E., and Lin, P S. Scanning electron microscopy and the surface morphology of human lymphocytes. Nature, Lond. *275* 15–18 (1978)

Received. November 6, 1978
Accepted. April 5 1979

J Renau-Piqueras, PhD,
Instituto de Investigaciones Citológicas,
Amadeo de Saboya 4 Valencia 10 (Spain)

Acta haemat. 62: 191-198 (1979)

Microbicidal Function of the Neutrophils in Hairy-Cell Leukaemia

J. A. Child, J. C. Cawley, S. Martin and A. T. M. Ghoneim

Departments of Haematology and Medicine, Leeds General Infirmary, and Department of Microbiology, School of Medicine, University of Leeds, Leeds

Key Words. Candida · Chronic lymphocytic leukaemia · Hairy-cell leukaemia · Neutrophil function · Splenectomy · Staphylococcus

Abstract. The ability of neutrophils from patients with hairy-cell leukaemia to kill *Candida guilliermondii* and *Staphylococcus aureus in vitro* was investigated. Of 10 patients, 8 showed defective candida killing, 5 defective bacteria killing, in comparison with normal controls. Study of those patients who underwent splenectomy suggests that neutrophil function can be improved by splenectomy. The possible causes of defective function in this disease are discussed.

Introduction

The clinical and pathological features of hairy-cell leukaemia (HCL) are now well defined. Pancytopenia is a characteristic feature and neutropenia is an important underlying cause of infection in these patients. In a recent study, the incidence of serious infections was 40% [3]. There is accumulating evidence to suggest that the microbicidal function of neutrophils in various haematological malignancies may be defective [5, 9, 10, 15] and in a recent study the neutrophils in patients with HCL showed an impaired candidacidal activity *in vitro* [8]. In view of this finding and the possibility that defective function could have clinical relevance, a group of 10 patients with HCL were studied in more detail.

Patients, Materials and Methods

HCL Patients

10 patients were studied: 3 were investigated before and after splenectomy; 4 had not undergone splenectomy and 3 were investigated only after splenectomy (table I).

The HCL patients fulfilled the accepted clinical, haematological, cytological and cytochemical criteria of the disease [2, 7]; in all instances the diagnosis was confirmed by histopathological examination of either the spleen or the bone marrow [19]. Relevant clinical and haematological data are set out in table I.

Normal Controls and Other Patients

In each test of patient neutrophil function, neutrophils from normal healthy volunteer subjects were studied in parallel. For comparison, 4 patients with untreated chronic lymphocytic leukaemia were studied: all had typical chronic lymphocytic leukaemia (CLL) with absolute lymphocyte counts $> 15 \times 10^9$/l and moderate to marked

splenomegaly In addition, the neutrophils from a patient with a high neutrophil alkaline phosphatase (NAP) score, comparable to that of our HCL patients, were studied this patient had neutrophilia secondary to pyogenic infection. Finally a patient with neutropenia comparable to that commonly observed in HCL was studied. this patient had Felty's syndrome and was investigated before and after splenectomy Relevant clinical and haematological data are set out in table I

Neutrophil Preparation

Heparinised blood samples were sedimented in dextran. Neutrophils were separated from leucocyte-rich plasma, using a discontinuous gradient of Ficoll-Triosil [11] washed twice and re-suspended to give 1×10^7 neutrophils/ml Hanks balanced salt solution containing 0 1 / gelatin (HBSS-gel) viability by trypan blue exclusion was > 95%.

Candidacidal Assay

This was adapted from the methods of *Lehrer* [20] and *El-Maalem and Fletcher* [10] The test organisms, *Candida guilliermondii* were washed twice and suspended to give a concentration of 3×10^7 candida/ml HBSS-gel. Viability by methylene blue exclusion was > 97%. The assay mixture contained 0.25 ml each of candida, neutrophils, HBSS-gel and pooled AB serum. The candida: neutrophil ratio was 3 1 Aliquots were removed at 90 min, smears prepared and stained with May Grünwald-Giemsa. The number of candida within at least 100 neutrophils were counted for viable, dark blue organisms and pale non viable ghosts' Results were expressed as percentage killing and also as a leucocyte candidacidal index (LCI) derived from the log% viable organisms at 90 min in each test:

$$LCI = \frac{\log\% \text{ viable } {}^{90\ min} \text{ unknown} - \log\% \text{ viable } {}^{90\ min} \text{ control}}{\text{normal standard deviation } {}^{90\ min}}$$

Where LCI is +2 or greater function is regarded as significantly impaired [8]

Bactericidal Assay

This was adapted from the method of *S Iberg* [23]. *Staphylococcus aureus* (NCTC 6571) was cultured overnight, washed twice in 0.9% saline and re-suspended in HBSS-gel, to give a final concentration of 10×10 colony-forming units/mL The assay mixture contained 0.3 ml HBSS-gel, 0 1 ml bacteria, 0.5 ml neutrophils and 0 1 ml pooled AB serum. 10 μl aliquots were removed at time intervals to determined the total number of viable bacteria. Results were expressed as percentage killing: differences of >20% (for % killing by control neutrophils – / killing by patient neutrophils) were considered significant.

Levamisole Studies

Preservative free crystalline levamisole (Janssen Pharmaceutical Ltd.) was dissolved in HBSS-gel and added to the test system (not pre-incubated with the neutrophil preparation) to give a final concentration of 1×10^{-7} *M* This concentration approximates to the *in vivo* blood levels produced by usual doses of the drug [24]

Results

Table I shows the results of neutrophil function tests in 8 of 10 patients candida killing was abnormal, while staphylococcal killing was defective in 5 cases. In the 3 patients studied pre and post-splenectomy (HR, AB AF) the ability to kill both *C. guilliermondii* and *S aureus* showed improvement after splenectomy In the case of candida killing, there was early partial improvement, which after several months became complete in the 2 patients who were followed up (AB and AF) with return of function to normal

3 of the 4 splenectomised patients (HD FB and WE) had defects in their candida killing. Of these 3 patients, 1 (HD) had significantly defective bacteria killing while in the other 2 (FB and WE) it was reduced, although not by a difference of > 20% in comparison with normal controls. 1 non-splenectomised patient (KD) showed normal killing function in both tests.

Of the 3 patients who were only studied after splenectomy (13–42 months following) 1 (PS) showed normal killing function. the other 2 patients (FW and LH) had defective candida and bacteria killing when

they were first tested, but in both cases killing function had improved to normal when the patients were re-tested at a later date. In 4 of the 8 patients who had abnormal candida killing, the addition of levamisole enhanced function to normal.

Table II shows results of neutrophil function tests in 4 patients with CLL, in 1 with Felty's syndrome and in 1 with a high NAP score. The patients with CLL and the patient with the high NAP score had normal candida and bacteria killing. The patient with Felty's syndrome had defective candida and bacteria killing, both of which improved to normal after the patient had been splenectomised.

Discussion

In the present study neutrophils from patients with HCL showed defective killing of *C guilliermondii in vitro* in 8 of the 10 patients *S aureus* killing was abnormal in 5 cases. The tests of microbicidal function adopted here were specifically designed to investigate intracellular killing and no attempt was made to assess plasma factors in the disease Reduced microbicidal function could not be attributed to drug therapy since most of the patients were untreated at the time of investigation and it seems probable that these *in vitro* abnormalities reflect significant derangement of comparable function *in vivo* and imply susceptibility to opportunistic infections.

Although qualitative neutrophil defects in HCL have not been described from other laboratories, impaired ability of neutrophils to kill micro-organisms *in vitro* has been reported in association with a variety of haematological malignancies, notably acute lymphoblastic leukaemia [15] acute myeloid and myelomonocytic leukaemias [9 14] pre-leukaemia [5] and chronic granulocytic leukaemia [10] The evidence for similar defects in CLL, the leukaemia which most closely resembles HCL, is less certain and bactericidal function has been variously reported as normal [4 18] or defective [18, 21] though there have been no recently published investigations of such patients using currently acceptable methods. In most leukaemias, neutropenia would seem to be usually of more clinical relevance than any possible qualitative defect. However our demonstration of improved neutrophil function *in all 3 HCL patients studied before* and after splenectomy may have important clinical implications, since splenectomy is the current major form of therapy in the disease [7 12, 17 22]

As to the nature and cause of the neutrophil defect in HCL, there are a number of aspects which deserve consideration. A characteristic abnormality of the neutrophils in HCL shown by all our patients is the presence of increased levels of neutrophil alkaline phosphatase [16] The exact role of alkaline phosphatase in the ingestion and killing of micro-organisms is not known. The low NAP score in CGL does not appear to be directly related to impaired candida phagocytosis and killing [10] The possibility that the *high* NAP score in HCL is in some way related to defective microbicidal activity requires further investigation of granulation and degranulation in these neutrophils. Our demonstration of normal neutrophil function in a patient without malignancy but with infection, neutrophilia and a high NAP score shows that there can be no simple relationship between a high score and defective function.

splenomegaly In addition, the neutrophils from a patient with a high neutrophil alkaline phosphatase (NAP) score, comparable to that of our HCL patients, were studied. this patient had neutrophilia secondary to pyogenic infection. Finally a patient with neutropenia comparable to that commonly observed in HCL was studied. this patient had Felty's syndrome and was investigated before and after splenectomy Relevant clinical and haematological data are set out in table I.

Neutrophil Preparation

Heparinised blood samples were sedimented in dextran. Neutrophils were separated from leucocyte-rich plasma, using a discontinuous gradient of Ficoll Triosil [11], washed twice and re-suspended to give 1×10^7 neutrophils/ml Hanks balanced salt solution containing 0.1 / gelatin (HBSS-gel) viability by trypan blue exclusion was > 95%.

Candidacidal Assay

This was adapted from the methods of *Lehrer* [20] and *El Maalem and Fletcher* [10] The test organisms, *Candida guilliermondii* were washed twice and suspended to give a concentration of 3×10^7 candida/ml HBSS-gel. Viability by methylene blue exclusion was > 97%. The assay mixture contained 0.25 ml each of candida, neutrophils, HBSS-gel and pooled AB serum. The candida neutrophil ratio was 3 1 Aliquots were removed at 90 min, smears prepared and stained with May-Grünwald-Giemsa. The number of candida within at least 100 neutrophils were counted for viable, dark blue organisms and pale non viable ghosts Results were expressed as percentage killing and also as a leucocyte candidacidal index (LCI) derived from the log% viable organisms at 90 min in each test:

$$LCI = \frac{\log\% \text{ viable }_{90 \text{ min}} \text{ unknown} - \log\% \text{ viable }_{90 \text{ min}} \text{ control}}{\text{normal standard deviation }_{90 \text{ min}}}$$

Where LCI is +2 or greater function is regarded as significantly impaired [8].

Bactericidal Assay

This was adapted from the method of *Solberg* [23] *Staphylococcus aureus* (NCTC 6571) was cultured overnight, washed twice in 0.9% saline and re-suspended in HBSS-gel, to give a final concentration of 10×10 colony-forming units/ml. The assay mixture contained 0.3 ml HBSS-gel, 0 1 ml bacteria, 0.5 ml neutrophils and 0 1 ml pooled AB serum. 10 μl aliquots were removed at time intervals to determined the total number of viable bacteria. Results were expressed as percentage killing: differences of >20% (for % killing by control neutrophils – % killing by patient neutrophils) were considered significant.

Levamisole Studies

Preservative-free crystalline levamisole (Janssen Pharmaceutical Ltd.) was dissolved in HBSS-gel and added to the test system (not pre-incubated with the neutrophil preparation) to give a final concentration of 1×10^{-7} *M* This concentration approximates to the *in vivo* blood levels produced by usual doses of the drug [24]

Results

Table I shows the results of neutrophil function tests. in 8 of 10 patients candida killing was abnormal, while staphylococcal killing was defective in 5 cases. In the 3 patients studied pre and post-splenectomy (HR, AB AF) the ability to kill both *C. guilliermondii* and *S aureus* showed improvement after splenectomy In the case of candida killing, there was early partial improvement, which after several months became complete in the 2 patients who were followed up (AB and AF) with return of function to normal

3 of the 4 splenectomised patients (HD FB and WE) had defects in their candida killing. Of these 3 patients, 1 (HD) had significantly defective bacteria killing while in the other 2 (FB and WE) it was reduced, although not by a difference of >20% in comparison with normal controls. 1 non-splenectomised patient (KD) showed normal killing function in both tests.

Of the 3 patients who were only studied after splenectomy (13–42 months following) 1 (PS) showed normal killing function the other 2 patients (FW and LH) had defective candida and bacteria killing when

% candida killing					% bacteria killing		
patient	control	difference	LCI	LCI + LMS	patient	control	difference
32	68	−36	5.0	ND	44	87	−43
ND	ND	ND	ND	ND	88	96	− 8
37	59	−22	2.9	−0.4	ND	ND	ND
ND	ND	ND	ND	ND	71	95	− 4
19	50	−31	3.2	ND	ND	ND	ND
51	70	−19	3.2	1.4	91	94	− 3
63	67	− 4	0.8	0.8	94	96	− 2
50	75	−25	4.6	4.0	84	95	−11
55	69	−14	2.6	1.9	79	78	+ 1
55	50	+ 5	−0.7	−0.7	ND	ND	ND
52	58	− 6	0.8	0.1	70	70	
14	58	−44	4.8	3.3	40	70	−30
47	69	−22	3.6	ND	72	89	−17
48	77	−29	5.4	2.6	82	93	−11
18	54	−36	3.4	1.9	55	75	−20
48	58	−10	1.4	−0.6	87	97	−10
59	54	+ 5	−0.8	−0.9	81	80	+ 1
22	51	−29	3.1	3.0	55	82	−27
62	58	+ 4	−0.6		90	97	− 7

Table I HCL patients

Patient	Date	Splenectomy	Spleen cm	Drugs	WBC × 10⁹/l	H. cells × 10⁹/l	Neuts 10⁹/l	NAP
H R.	9 10 77	10.10.77	17	pred. 40 mg/day	9 8	5 4	0.9	200
	18 10.77		–	pred. 20 mg/day	7.0	[illegible]0	4.3	210
	20.10.77		–	pred. 15 mg/day	5 1	0.9	[illegible]7	220
A B	25 10 77		7	nil	3 0	0.7	0.3	ND
	31 10.77		7	nil	4 1	0.8	0 5	ND
		21 11 77						
	16.12.77		–	nil	4.2	0 7	1 1	151
	4 7 78		–	nil	4.9	0.4	0.8	ND
A.F	19 7 78		6	nil	4.8	1 7	0.48	250
		26. 7 78						
	3 8 78		–	nil	5 1	1.2	0.51	268
	21 9 78		–	nil	30.0	19.5	1 8	ND
K.D	13 4 78		12	nil	10.1	6.2	1 8	[illegible]
H D	13 4 78		–	pred. 10 mg day	3 7	0 85	0.96	10
F.B	11 8.78		3	pred. 10 mg/day	3 7	1.25	0.96	180
W.E.	19 10 78		16	nil	14.2	9 9	0.57	220
F W		31 8 74						
	21 2.78		–	nil	4.2	0.8	0.26	ND
	18. 7 78		–	nil	4 6	0.78	3 1	[illegible]
P.S.		19 10.76						
	23 2.78		–	nil	7.5	2.0	3.3	260
		1[illegible] 1 77						
L.H.	24 2.78		–	nil	3.9	0.14	[illegible]18	ND
	18 7 78		–	nil	3 7	0.36	0.77	290

HCL = Hairy-cell leukaemia CLL = chronic lymphocytic leukaemia NAP = neutrophil alkaline phosphatase H. cells = hairy cells LCI = leucocyte candidacidal index LMS = levamisole.

% candida killing					% bacteria killing		
patient	control	difference	LCI	LCI + LMS	patient	control	difference
32	68	−36	5.0	ND	44	87	−43
ND	ND	ND	ND	ND	88	96	− 8
37	59	−22	2.9	−0.4	ND	ND	ND
ND	ND	ND	ND	ND	71	95	−24
19	50	−31	3.2	ND	ND	ND	ND
51	70	−19	3.2	1.4	91	94	− 3
63	67	− 4	0.8	0.8	94	96	− 2
50	75	−25	4.6	4.0	84	95	−11
55	69	−14	6	1.9	79	78	+ 1
55	50	+ 5	−0.7	−0.7	ND	ND	ND
52	58	− 6	0.8	0.1	70	70	
14	58	−44	4.8	3.3	40	70	−30
47	69	−22	3.6	ND	72	89	−17
48	77	−29	5.4	2.6	82	93	−11
18	54	−36	3.4	1.9	55	75	−20
48	58	−10	1.4	−0.6	87	97	−10
59	54	+ 5	−0.8	−0.9	81	80	+ 1
22	51	−29	3.1	3.0	55	82	−27
62	58	+ 4	−0.6		90	97	− 7

Table II Other patients

Patient	Date	Splenectomy	Spleen cm	Drugs	WBC × 10⁹/l	Neuts 10⁹/l	NAP
CLL							
P.B	8 5 78		6	nil	28.[illegible]	8 4	125
	[illegible]6. 6.78		6	nil			
H.A.	9 5 78		8	nil	49 0	4 6	96
M W	7 8 78		14	nil	17.2	4.3	104
W C.	3 11 78		tip	nil	53 0	6.4	124
Felty s syndrome							
J L.	4 5 78		4	pred.	1.3	0.18	112
		18. 5 78		7.5 mg/day			
	1 6.78		–	nil	7.2	3.5	ND
High NAP							
J W		15 11 77		gentamicin frusemide			
	21 12.77		–	benzyl-penicillin	78 8	26.5	219

The 4 untreated patients with CLL and splenomegaly provided another interesting comparison none showed any evidence of defective function. However unlike the patients with HCL, their neutrophil counts were normal and this raised the question of whether or not the functional defects in HCL were a consequence of neutropenia *per se* In our experience we have found abnormal microbicidal tests in patients with haematological malignancies and normal or elevated absolute counts as well as in those with neutropenia [unpubl. observations] That splenomegaly is of significance in the neutrophil dysfunction of HCL, however seems very likely in view of the changes observed in relation to splenectomy In the 3 patients studied before and after splenectomy candida killing was improved by this procedure Of 3 previously splenectomised patients, 2 had abnormal candida killing and 1 abnormal bacteria killing tests, all of which subsequently reverted to normal in the course of follow up In the 1 patient with non malignant splenomegaly who we were able to study before and after splenectomy abnormal candida and bacteria killing were both corrected following splenectomy

The addition of levamisole *in vitro* normalised candida killing in 4 patients, all of whom had been splenectomised in 2 non-splenectomised and 1 splenectomised patient function was not normalised The significance of the levamisole effect [8] is not clear but there is evidence to suggest that levamisole may maintain or increase cyclic guanosine monophosphate (cGMP) [1] and this could enhance microtubular assembly and degranulation

There is now overwhelming evidence that HCL is a form of B-cell proliferation [6 7 13] and there would appear to be little

% candida killing					% bacteria killing		
patient	control	difference	LCI	LCI + LMS	patient	control	difference
63	62	1	−0.1	ND	57	61	−4
62	70	−8	1.6	ND	92	83	9
50	50				74	78	−4
73	70	3	−0.7	−0.5	ND	ND	ND
57	67	−10	1.8	ND	91	95	−4
24	52	−28	3.0	2.7	29	66	−37
69	69			ND	84	89	−5
60	50	10	−1.4	−1.9	90	87	3

supporting evidence pointing to a primary defect of the myeloid series in HCL. Our demonstration of normal neutrophil function in CLL, a closely related B-cell tumour supports this belief.

It is tempting to speculate that the tendency for neutrophil function to improve following splenectomy relates to the preferential pooling of the more functionally competent neutrophils in the spleen. Another possibility is that quantitatively abnormal granulocyte production, particularly in patients with bone marrow compromised by infiltration or fibrosis, is associated with intrinsic qualitative abnormalities. Bearing in mind the fact that apparently similar function defects may occur in a variety of disease states, it would appear likely that there are several possible underlying causes of such abnormalities. Whether or not there is a single cause of this derangement in HCL, our findings in these patients suggest that defective microbicidal function may be an additional factor in favour of splenectomy.

Acknowledgements

Mrs. *S. Martin* is supported by grants from the Endowment Fund, Leeds General Infirmary and Janssen Pharmaceutical Ltd. We are also grateful to Professor *E. Mary Cooke*, Professor *G. P. McNicol*, Dr. *B. E. Roberts* and Dr. *R. Sibbald* for their co-operation in this study.

References

1 Anderson, R., Glover, A., Koornhof, H. J. and Rabson, A. R.: *In vitro* stimulation of neutrophil motility by levamisole: maintenance of cGMP levels in chemotactically stimulated levamisole-treated neutrophils. J. Immun. *117*: 428–432 (1976).

Bouroncle, B. A., Wiseman, B., and Doan, C.

A. Leukemic reticuloendotheliosis. Blood *13* 609–630 (1958).

3 Bouza, E. Burgaleta, C., and Golde, D W.. Infections in hairy-cell leukaemia. Blood *51* 851–859 (1978)

4 Braude, A. I Feltes, J and Brooks, M Differences between the activities of mature granulocytes in leukemic and normal blood. J clin. Invest. *33* 1036–1046 (1954)

5 Breton-Gorius, J Houssay D., Vilde, J L., and Dreyfus, B.. Partial myeloperoxidas deficiency in a case of preleukaemia. II Defects of degranulation and abnormal bactericidal activ ity of blood neutrophils. Br J Haemat. *30* 279–288 (1975).

6 Burns, G F., Cawley J C. Worman, C. P Barker C. R. Edwards, M Rees, J K. H., and Hayhoe, F G J Hairy-cell leukemia. a B-cell neoplasm with severe deficiency of nor mal B lymphocytes. Leukemia Res. *2* 33–40 (1977).

7 Catovsky D Hairy-cell leukaemia and pro-lymphocytic leukaemia. Clin. Haematol *6*. 245–268 (1977).

8 Child, J A. Martin, S. Cawley J C., and Ghoneim, A. T M Defective microbicidal function of neutrophils in haematological ma lignancies and lymphomas: correction by lev amisole *in vitro* Biomedicine *29* 159–161 (1978)

9 Davis, A. T Brunning, R. D and Quie, P G Polymorphonuclear leukocyte myeloperox idase deficiency in a patient with myelomonocytic leukaemia. New Engl. J Med. *285* 789–791 (1971)

10 El Maslem, H and Fletcher J Defective neutrophil function in chronic granulocytic leukaemia. Br J Haemat. *34* 95–103 (1976).

11 English, D and Anderson, B R.. Single-step separation of red blood cells, granulocytes and mononuclear leucocytes on discontinuous density gradients of Ficoll-Hypaque. J immunol. Methods *5* 249–252 (1974).

12 Feiner A. S., Myers, A. M and Moore, G E.. Leukaemic reticuloendotheliosis. Loss of platelet defect after splenectomy J Am. med. Ass. *240* 238–40 (1978).

13 Golde, D W., Stevens, R. H., Quan, S. G and Saxon, A.. Immunoglobulin synthesis in hairy cell leukaemia. Br J Haemat. *35* 359–361 (1977)

14 Goldman, J M. and Th ng, K. H.. Phagocytic function of leucocytes from patients with acute myeloid and chronic granulocytic leukaemia. Br J Haemat. *25* 299–308 (1973).

15 Gregory L. Williams, R., and Thompson, E Leucocyte function in Down s syndrome and acute leukaemia. Lancet *i*. 1359–1361 (1972).

16 Hayhoe, F G J Flemans, R. J Burns, G. F and Cawley J C.. Leucocyte alkaline phosphatase scores in hairy-cell leukaemia. Br J. Haemat. *37* 158–159 (1977).

17 Jansen, J Hermans, J., Remme, J Ottolander G J den, and Lopes Cardoza, P Hairy cell leukaemia. clinical features and effect of splenectomy Scand. J Haematol. *21* 60–71 (1978)

18 Kallinske, R. W and Hoeprich, P D Engulfment and bactericidal capabilities of peripheral blood leukocytes in chronic leukaemias. Cancer *23* 1094–1102 (1969).

19 Katayama, I. Hairy-cell leukemia. New Engl J Med. *296*. 881 (1977)

20 Lehrer R. I Measurement of candidacidal activity of specific leucocyte types in mixed cell populations. I Normal, myeloperoxidase-deficient and chronic granulomatous disease neutrophils. Infect. Immun *2* 42–47 (1970).

21 Sbarra, A. J Shirley W Selvaraj, R. J., Ouchi, E., and Rosenbaum, E.. The role of the phagocyte in host-parasite interaction. I. The phagocytic capabilities of leukocytes from lymphoproliferative disorders. Cancer Res. *24* 1958–1968 (1964)

22 Sebahoun, G Boufette, P and Flandrin, G.. Hairy-cell leukemia. Leukemia Res. *2*. 187–195 (1978)

23 Solberg, C. O Evaluation of neutrophil granulocyte functions. Acta. path. microbiol scand. *80* 559–563 (1972)

24 Symoens, J Levamisole, n a tianergic chemotherapeutic agent an overview in Chirigos, Control of neoplasia by modulation of the immune system, p 24 (Raven Press, New York 1977).

Received. January 12, 1979
Accepted April 23, 1979

J A. Child, MD MRCP Department of Haematology Leed General Infirmary Leeds LS1 3EX (Engl d)

Acta haemat. 62: 199–205 (1979)

Terminal Transferase in Leukemia of Adults

G. A. Pangalis and E. Beutler

Department of Hematology, City of Hope National Medical Center, Duarte, Calif.

Key Words. Terminal deoxynucleotidyl transferase · Adult leukemia · Acute nonmyelocytic leukemia · Blastic crisis of chronic myelogenous leukemia · Chemotherapy response

Abstract. Terminal deoxynucleotidyl transferase (TdT) determinations were carried out on peripheral blood leukocytes or bone marrow cells from 61 adult patients with various types of leukemia. TdT activity was undetectable in the cells of patients with acute myelocytic or acute myelomonocytic leukemia but was present in 12 of 13 patients with acute nonmyelocytic leukemia. None of 3 patients with acute myelocytic transformation of chronic myelocytic leukemia (CML) manifested TdT activity while 4 of 6 patients with lymphoid transformation had such activity.

More patients with TdT in their leukemic cells responded to treatment than those without TdT activity. However, our findings suggest that TdT activity may be less useful in management of leukemia than has sometimes been supposed.

Introduction

The acute myelocytic, myelomonocytic and monocytic leukemias in adults possess certain distinct morphologic and cytochemical characteristics [11, 12]. In contrast, morphology and cytochemistry are of limited value in the diagnosis of acute lymphoblastic leukemia (ALL) since the diagnosis is based largely on negative observations such as lack of granule differentiation or Sudan black B, peroxidase and naphthol AS-D chloroacetate esterase positivity in the blast cells [11]. Although some cases of acute leukemia can positively be diagnosed immunologically as ALL by the use of specific antisera, E-rosettes and surface immunoglobin studies [9], other patients with acute leukemias remain unclassifiable [13]. Because the myeloblastic or lymphoblastic nature of a leukemic cell population is important in the selection of appropriate therapy, the use of additional markers for more accurate differentiation between lymphoblastic and myeloblastic leukemias is potentially useful. Terminal deoxynucleoti-

A. Leukemic reticuloendotheliosis. Blood *13* 609–630 (1958)
3 Bouza, E. Burgaleta, C., and Golde, D W Infections in hairy-cell leukaemia. Blood *51* 851–859 (1978)
4 Braude, A. I. Feltes, J and Brooks, M. Differences between the activities of mature granulocytes in leukemic and normal blood. J clin. Invest. *33* 1036–1046 (1954)
5 Breton-Gorius, J Houssay D Vilde, J L., and Dreyfus, B Partial myeloperoxidase deficiency in a case of preleukaemia. II. Defects of degranulation and abnormal bactericidal activity of blood neutrophils. Br J Haemat. *30* 279–288 (1975).
6 Burns, G F Cawley J C. Worman, C. P Barker C. R., Edwards, M Rees, J K. H., and Hayhoe, F G J Hairy-cell leukemia. a B-cell neoplasm with severe deficiency of normal B lymphocytes. Leukemia Res. *2* 33–40 (1977).
7 Catovsky D Hairy-cell leukaemia and prolymphocytic leukaemia. Clin. Haematol. *6* 245–268 (1977)
8 Child, J A. Martin, S. Cawley J C., and Ghoneim, A. T M Defective microbicidal function of neutrophils in haematological malignancies and lymphomas. correction by levamisole *in vitro* Biomedicine *29* 159–161 (1978)
9 Davis, A. T Brunning, R. D and Quie, P G Polymorphonuclear leukocyte myeloperoxidase deficiency in a patient with myelomonocytic leukaemia New Engl. J Med. *285* 789–791 (1971).
10 El Maalem, H and Fletcher J Defective neutrophil function in chronic granulocytic leukaemia. Br J H emat. *34* 95–103 (1976)
11 English, D and Anderson, B. R. Single-step separation of red blood cells, granulocytes and mononuclear leucocytes on discontinuous density gradients of Ficoll Hypaque. J immunol. Methods *5* 249–252 (1974).
12 Feiner A. S Myers, A. M and Moore, G E. Leukaemic reticuloendotheliosis. Loss of platelet defect after splenectomy J Am. med. Ass. *240* .38–240 (1978)
13 Golde, D W Stevens, R. H. Quan, S G and Saxon, A. Immunoglobulin synthesis in hairy cell leukaemia. Br J Haemat. *35* 359–361 (1977)
14 Goldman, J M. and Th'ng, K. H. Phagocytic function of leucocytes from patients with acute myeloid and chronic granulocytic leukaemia. Br J Haemat. *25* 299–308 (1973).
15 Gregory L., Williams, R., and Thompson, E. Leucocyte function in Down's syndrome and acute leukaemia. Lancet *I*. 1359–1361 (1972).
16 Hayhoe, F G J., Flemans, R. J Burns, G. F and Cawley J C. Leucocyte alkaline phosphatase scores in hairy-cell leukaemia. Br J Haemat. *37* 158–159 (1977).
17 Jansen, J Hermans, J Remme, J Ottolander G J den, and Lopes Cardoza, P Hairy cell leukaemia. clinical features and effect of splenectomy Scand. J Haematol. *21* 60–71 (1978)
18 Kalinske, R. W and Hoeprich, P D Engulfment and bactericidal capabilities of peripheral blood leukocytes in chronic leukaemias. Cancer *23* 1094–1102 (1969).
19 Katayama, I Hairy-cell leukemia. New Engl. J Med. *296*. 881 (1977)
20 Lehrer R. I Measurement of candidacidal activity of specific leucocyte types in mixed cell populations. I. Normal, myeloperoxidase-deficient and chronic granulomatous disease neutrophils. Infect. Immun *2*. 42–47 (1970).
21 Sbarra, A. J Shirley W Selvaraj, R. J Ouchi, E., and Rosenbaum, E.. The role of the phagocyte in host-parasite interaction. I The phagocytic capabilities of leukocytes from lymphoproliferative disorders. Cancer Res. *4* 1958–1968 (1964)
22 Sebahoun, G Boufette, P and Flandrin, G. Hairy-cell leukemia. Leukemia Res. *2*. 187–195 (1978).
23 Solberg, C. O Evaluation of neutrophil granulocyte functions. Acta path. microbiol. scand. *80* 559–563 (1972).
24 Symoens, J Levamisole, an antianergic chemotherapeutic agent: an overview. In Chirigos, Control of neoplasia by modulation of the immune system, p. .4 (Raven Press, New York 1977)

Received January 12, 1979
Accepted April 23, 1979

J A. Child, MD MRCP Department of Haematology Leeds General Infirmary Leeds LS1 3EX (England)

Materials and Methods

61 newly diagnosed adult patients with leukemia and 6 normal bone marrow donors were included in this study. The median age of the patients was 45 years with range of from 18 to 68. Age, sex, date of diagnosis, clinical presentation, hematologic findings, type of chemotherapy given, response to treatment, and the patient's clinical course were evaluated in every case. Karyotype analysis was performed in all cases in which the diagnosis of CML was suspected.

Table I. Terminal deoxynucleotidyl transferase activity in adult leukemias

Diagnosis	Number of cases	Number of cases with TdT activity	TdT activity μU/mg protein
Acute leukemias			
Myelocytic (AML)	11	0	
Myelomonocytic (AMML)	4	0	
Nonmyelocytic (ANML)	13	12	24–1,632
Chronic leukemias			
Myelocytic (CML)	7	0	
Myelocytic in blastic crisis (myeloblasts)[1]	3	0	
Myelocytic in blastic crisis (nonmyeloblasts)	6	4	100–2,090
Prolymphocytic			
Lymphocytic of T cell	3	0	
type	1	0	
Smouldering leukemia (refractory anemia with excess of blasts)	11	2	49 and 267
Others			
Plasma cell leukemia	1	0	
Immunoblastic leukemia [18]	1	0	

[1] As determined by the Sudan black B, peroxidase and naphthol AS-D chloroacetate esterase reactions.

TdT Determination

7 ml of blood or 1-2 ml of bone marrow samples were collected before treatment into tubes containing 7 mg EDTA. The mononuclear cells were isolated using the Ficoll-Hypaque technique and the TdT activity was determined as described previously [3].

Morphologic and Cytochemical Determinations

Blood or bone marrow films from each patient were stained as follows: May-Grünwald-Giemsa, periodic acid-Schiff (PAS) [12], Sudan black B (SBB) [12], peroxidase [12], esterase with naphthol As-di-chloroacetate as substrate (NCA), esterase with naphthol AS-D acetate as substrate (NAS-DA), and NASDA with sodium fluoride [8]. In each case, differential count was carried out and the percentage of blast cells giving positive reaction to each of the cytochemical reactions was recorded. Acute leukemias with more than 5% SBB, peroxidase, or NCA-positive blast cells, were diagnosed as AML [11] or if the NASDA reactivity was not inhibited by sodium fluoride, as myelomonocytic leukemia (AMML) [8]. Those of the patients in which the blast cells were negative in the SBB, peroxidase and NCA reactions are designated as acute nonmyelocytic leukemia (ANML); the blast cells of such patients manifested various degrees of PAS reactivity in the form of cytoplasmic granules.

The diagnosis of refractory anemia with excess of blasts (smouldering leukemia) was based on previously reported criteria [26]. The blast cells in these patients were SBB and peroxidase positive.

Results

Normal Bone Marrow TdT Activity. The TdT level of the six normal bone marrow samples studied ranged from negative to 15 μU/mg protein (fig. 1).

TdT Activity in Leukemias. The results of TdT assays are summarized in table I and figure 1. The cells of the 15 patients with AML or AMML were found to have no detectable TdT activity. In contrast those

dyl transferase (TdT) discovered in the early 1960s [2, 15] may serve such a purpose TdT catalyses, without presence of template, the addition of deoxynucleosides to poly deoxynucleotide chains The biologic role of this enzyme is unknown High TdT levels have been found in the normal thymus [6 19] and a trace of enzyme activity has been detected in cells from normal bone marrow [1 6 10 14] Increased TdT activity *has been reported to be present in almost* all cases of ALL [3 5 6 10 13 14 16 19 20] as well as in a number of cases of chronic myelocytic leukemia (CML) in blastic crisis [3 5 6 13 14 17 19 23 24] in lymphoblastic lymphoma [7 16] and in rare cases of acute myelocytic leukemia (AML) [3 25]

We have now investigated the activity of bone marrow or peripheral blood TdT in a large number of adults with leukemia, with particular emphasis on the group of patients classified as acute nonmyelocytic (ANML) by cytochemical criteria. We have addressed, *also the question of* whether TdT activity has any value in assessing response to chemotherapy

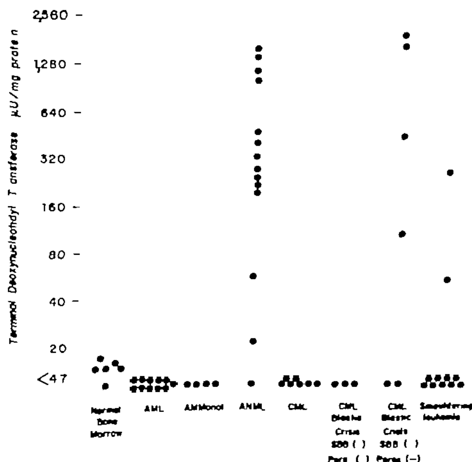


Fig. 1. Terminal deoxynucleotidyl *transferase activity of marrow or peripheral blood cells partially purified as indicated in the text.*

Table III. Chronic myelocytic leukemia TdT activity and response to treatment

Case No	Age	Source of cells	WBC 10^3	Blast cells %	Number of blast cells 10^9	Cytochemistry % of positive cells SBB	PEROX	TdT activity µU/mg	Chemotherapy	Response
1	41	blood	308.0	81	249.5	70	75	< 4.8	VCR, PRE	NR
2	23	blood	253.0	55	139.1	15	10	< 4.8	VCR, PRE ADRIA, Ara-C	NR NR
3	63	BM		60	–	100	100	< 4.8	Hydrea	control of patient's leukocytosis
4	52	BM		40		0	0	2,020.8	VCR PRE 6MP	PR
5	46	BM		30		0	0	100.0	VCR PRE 6MP	PR
6	35	blood	149.0	59	88.0	0	0	4.8	VCR, PRE, TG	PR
7[1]	30	blood	40.0	40	96.0	5	5	< 4.8	VCR, PRE TG Ara-C	PR
8	51	BM		100		0	0	2,090.0	VCR, PRE	PR
9	59	BM		100		0	0	431.9	VCR, PRE	PR

TdT = Terminal deoxynucleotidyl transferase SBB = Sudan black B PEROX = peroxidase BM = bone marrow VCR = vincristine PRE = prednisone 6MP = 6-mercaptopurine ADRIA = adriamycin Ara-C = cytosine arabinoside TG = thioguanine Hydrea = hydroxyurea PR = Significant decrease (> 50%) of WBC and number of blast cells in the blood and the bone marrow with or without reduction of the size of the spleen.

[1] This patient presented in blastic crisis.

low and undetectable TdT were represented. A complete remission was achieved in the remainder of the patients with ANML although in 2 of them, this was accomplished only with second-line combination chemotherapy after failure of conventional anti-ALL chemotherapy which included vincristine-prednisone (table II). In the group of patients with CML in blastic crisis, a partial response to vincristine and prednisone was observed in all SBB and peroxidase-negative cases, 4 of whom also had detectable TdT activity (table III)

Discussion

TdT was present in the leukocytes of all but 1 of our cases of adult ANML. This group of patients with ANML is of particular interest because the diagnosis of ALL in adults is occasionally made with some concern and reservation. The fact that cells from 12 of 13 patients in this group were TdT positive, indicates that the leukemic cells of almost all ANML cases studied was related to the lymphoid cell line. 10 of these patients manifested moderate or high level

Table II. Acute nonmyelocytic leukemia, TdT activity in comparison with number of blast cells, and response to therapy

Case No.	Age	Source of cells	WBC × 10³	Blast cells, %	Number of blast cells × 10³	TdT activity μU/mg	Chemotherapy	Response
1	40	blood	20.0	50	10.0	1,629.2	VCR, PRE	CR
2	18	blood	22.7	33	7.5	366.8	VCR, PRE, DAUNO	NR
							CODA	CR
3	19	blood	4.7	38	1.8	237.7	VCR, PRE	CR
4	4	blood	30.4	50	15.2	248.8	COAP	CR
5	31	blood	140.0	80	112.0	1,181.4	VCR, PRE	NR
		BM		100		1,632.4	DATPO	CR
6	53	blood	2.7	20	0.5	58.5	CODA	NR
							COAP	NR
							MTX + 6MP	NR
7	19	blood	81.2	83	67.4	1,047.5	VCR, PRE	CR
8	52	BM	–	40	–	23.9	VCR, PRE	NR
							VCR, PRE, DAUNO	NR
							DATPO	NR
9	20	blood	28.0	99	27.7	320.1	CYT, VCR, PRE	CR
10	40	BM	–	60		228.2	VCR, PRE, L-Aspa	CR
11	68	blood	7.3	43	3.1	< 4.8	DATPO	NR
12	65	blood	338.0	97	327.8	985.5	VCR, PRE	NR
13	29	blood	10.0	82	8.2	293.4	CODA	CR

TdT = Terminal deoxynucleotidyl transferase. BM = bone marrow. VCR = oncovin. PRE = prednisone. L-Aspa = L-asparaginase. DAUNO = daunorubicin. MTX = methotrexate. CYT = cyclophosphamide. 6MP = 6-mercaptopurine. CR = complete response. NR = no response. CODA = cytosine arabinoside, oncovin, daunorubicin, and azacytosine. COAP = cytoxan, oncovin, cytosine arabinoside, prednisone. DATPO = daunorubicin, cytosine arabinoside, 6-thioguanine, prednisone, oncovin. COPP = cyclophosphamide, oncovin, procarbazine, prednisone.

from 12 of the 13 patients with ANML had TdT activity ranging from 24 to 1,632 μU/mg. Those with absent or low enzyme activity were the oldest patients in the ANML group; no relationship between PAS reactivity and TdT activity was observed. Cells from only 2 of 11 patients with smouldering leukemia manifested detectable TdT activity, 49 and 267 μU/mg protein, respectively (table II). All patients with CML were characterized by the presence of a Ph' chromosome. The leukocytes of 3 patients in blastic crisis, with SBB and peroxidase reactive blasts also had no detectable TdT activity. The 5 patients with CML in the chronic phase had no detectable TdT activity. In contrast, of the 6 patients with CML in blastic crisis in whom the blast cells had a lymphoid appearance [4, 22] and were SBB or peroxidase negative, 4 had TdT activity ranging from 100 to 2,090 μU/mg protein (table III).

Relationship between TdT Activity and Response to Therapy. 4 of the patients with ANML activity failed to respond to combination chemotherapy; subjects with high,

8 Flandrin, G et Daniel, M-T Cytochimie des estérases en hématologie. Pathol. Biol. 19 547–555 (1971).
9 Greaves, M. F Janossy G Roberts, M., et al. Membrane phenotyping: diagnosis, monitoring and classification of acute lymphoid leukemias; in Thierfelder and Rodt, Immunological diagnosis of leukemias and lymphomas, pp 61 75 (Berlin, 1977).
10 Greenwood, M. F Coleman, M. S Hutton, J J Lapkin, B Krill, C., Bollum, F J and Holland P Terminal deoxynucleotidyl-transferase distribution in neoplastic and hematopoietic cells. J clin Invest 59 889–899 (1977).
11 Hayhoe, F G J and Cawley J C. Acute leukemia. cellular morphology cytochemistry and fine structure. Clin Haematol 1 49-94 (1972).
12 Hayhoe, F G J Quaglino, D and Doll, R. The cytology and cytochemistry of acute leukemias. A study of 140 cases. MRC Special Report Series, No 304 (Her Majesty's Stationery Office, London 1964)
13 Hoffbrand, A. V Ganeshaguru, K., Janossy G Greaves, M. F Catovsky D and Woodruff, R. K Terminal deoxynucleotidyl-transferase levels and membrane phenotypes in diagnosis of acute leukemia. Lancet ii 520–523 (1977).
14 Hutton, J J and Coleman, M. S. Terminal deoxynucleotidyl transferase measurements in the differential diagnosis of adult leukaemias. Br J Haemat 34 447–456 (1976).
15 Krakow J S Coutsogeorgopoulos, C., and Canellakis, E S Studies on the incorporation of deoxyribonucleic acid. Biochim. biophys. Acta 5 639–650 (1962)
16 Kung, P C., Long, J C., McCaffrey R P Ratliff, R L Harrison, T A and Baltimore, D Terminal deoxynucleotidyl transferase in the diagnosis of leukemia and malignant lymphoma. Am. J Med. 64 788–794 (1978).
17 Marks, S. M Baltimore, D and McCaffrey R Terminal transferase and response to vincristine-prednisone in blastic chronic myelogenous leukemia New Engl. J Med 298 812–814 (1978)
18 Mathe, G Delpomme, D Dantchev D Pouillart, P Jasmin, C L, Misset, J L., Musset, M., Amiel, J L., Schlumberger, J R., Schwarzenberg, L., Hayat, M., DeVassal, F and La Fleur M. Immunoblastic acute lymphoid leukemia, an undescribed type. Biomedicine 20 333–340 (1974).
19 McCaffrey R., Harrison, T A., Parkman, R., and Baltimore, D Terminal deoxynucleotidyl transferase activity in human leukemic cells and in normal human thymocytes. New Engl. J Med. 292. 775–804 (1975).
20 McCaffrey R., Smoller D. F and Baltimore, D Terminal deoxynucleotidyl transferase in case of childhood acute lymphoblastic leukemia. Proc. natn. Acad. Sci. USA 70 521 525 (1973).
21 Murphy S B and Mauer A M Terminal transferase and lymphoblastic neoplasms. New Engl. J Med. 297 502 503 (1977).
22 Rosenthal, S Canellos, G P Whang-Peng, J and Gralnick, H R. Blastic crisis f chronic granulocytic leukemia Morphologic variants and therapeutic implications. Am. J Med. 63 542 547 (1977)
23 Saffhill, R., Dexter T M Muldal, S Testa, N G., Morris-Jones, P and Joseph, A., Terminal deoxynucleotidyl transferase in case of pH-positive infant chronic myelogenous leukemia. Br J Cancer 33 664–667 (1976).
24 Sarin, P S Anderson, P and Gallo, R. C.: Terminal deoxynucleotidyl transferase activities in human blood leukocytes and lymphoblast cell lines: high levels in lymphoblast cell lines and in blast cells of some patients with chronic myelogenous leukemia in acute phase. Blood 47 11–20 (1976).
25 Srivastava, B. I. S., Khan, S. A., and Henderson, E. S High terminal deoxynucleotidyl transferase activity in acute myelogenous leukemia. Cancer Res. 36 3847–3850 (1976).
26 Sultan, C. Dysmyelopoietic syndromes, pp 749–752, in Gralnick (moderator): Classification of acute leukemia. Ann intern. Med. 87 740–753 (1977).

Accepted: May 4, 1979

Ernest Beutler, MD,
Department of Clinical Research
Scripps Clinic and Research Foundation
10666 North Torrey Pines Road,
La Jolla, CA 92037 (USA)

of TdT activity while in two the enzyme activity was found to be very low (table II). In relation to the TdT level and response to therapy it was observed that most of the responders had a moderate to high enzyme level while most of the nonresponders had low or no enzyme activity (table II). Whether the TdT level of the blast cells of ANML patients has any direct implications in the therapeutic response of this group needs to be further investigated in a larger group of similar patients.

In CML and in CML in SBB and peroxidase positive blastic crisis no TdT activity was found, as has previously been reported [24]. In CML in SBB and peroxidase-negative blastic crisis, in which the blast cells had a lymphoid appearance, TdT activity was found in 4 of the 6 cases studied. A partial response was achieved in all 6 cases after administration of vincristine and prednisone (table III), an observation which is in agreement with previous reports [17, 22]. According to our results, however, it is possible that in CML in blastic crisis cytochemistry may be quite adequate in identifying those patients who are likely to respond to vincristine and prednisone.

TdT activity was found in 2 of the 11 patients with smouldering leukemia (refractory anemia with excess of blasts). In both patients, approximately 50% of the blast cell population was SBB and peroxidase reactive. We cannot be certain that the remainder of the blast cell population in these 2 cases were also myeloblastic in spite of the negative SBB and peroxidase reactions, or whether these blasts represent a separate lymphoid cell population as has been already pointed out for a few cases of CML in blastic crisis in which two different blast cell populations were identified [12].

In summary we find that the TdT estimation parallels the cytochemical findings both in acute leukemia and in the acute transformation of CML. Concerning the clinical significance of TdT determination [13, 21], we observed that in the ANML cases the TdT activity is an adequate guide to choose the appropriate therapeutic agents and that its level has no direct effect concerning the response to therapy. In the acute phase of CML, determination of TdT activity had no advantage over cytochemical markers in our patients.

References

1 Barr, R. D., Sarin, P. S., and Perry, S. M.: Terminal transferase in human bone-marrow lymphocytes. Lancet *i*: 508–509 (1976).

2 Bollum, F. J.: Oligodeoxyribonucleotide-primed reactions catalyzed by calf thymus polymerase. J. biol. Chem. *237*: 1945–1949 (1962).

3 Beutler, E. and Kuhl, W.: An assay for terminal deoxynucleotidyl transferase in leukocytes and bone marrow. Am. J. clin. Path. *70*: 733–737 (1978).

4 Canellos, G. P., DeVita, V. T., Whang-Peng, J. and Carbone, P. P.: Hematologic and cytogenetic remission of blastic transformation in chronic granulocytic leukemia. Blood *38*: 671–679 (1971).

5 Coleman, M. S., Greenwood, M. F., Hutton, J. J., Bollum, F. J., Lampkin, B., and Holland, P.: Serial observations on terminal deoxynucleotidyl transferase activity and lymphoblast surface markers in acute lymphoblastic leukemia. Cancer Res. *36*: 120–127 (1976).

6 Coleman, M. S., Hutton, J. J., DeSimone, P. and Bollum, F. J.: Terminal deoxyribonucleotidyl transferase in human leukemias. Proc. natn. Acad. Sci. USA *71*: 4404–4408 (1974).

7 Donlon, J. A., Jaffé, E. S., and Braylan, R. C.: Terminal deoxynucleotidyl transferase activity in malignant lymphomas. New Engl. J. Med. *297*: 461–464 (1977).

plasia (AAMM) is in our opinion preferable to that of AMF since bone marrow fibrosis is usually scanty in the latter whereas extra medullary hemopoiesis is a more prominent finding in the former [9]

In the ill-defined pancytopenias and myeloproliferative disorders, including a specific entity as polycythemia vera, chromosomal abnormalities are relatively common. The appearance of a clone of cells bearing a definite chromosomal abnormality represents a severe prognostic sign in certain preleukemic states, such as the pancytopenias [11] other myeloproliferative disorders, including acute myelofibrosis, should be added to the former since chromosomal alterations usually indicate that leukemia is already present [13]

Chronic myelofibrosis and polycythemia vera should be distinguished from these, since the appearance of aberrant clones does not seem to severely influence short term prognosis, while the clinical course is quite variable [4–6 8, 12, 13 15 20] Trisomy of chromosome No 8 was reported in 2 cases of AMF this peculiar chromosomal abnormality is extremely common in a variety of myeloid disorders, both preleukemic and frankly leukemic [10 12, 14 16] However chromosome No. 8 was apparently not involved in a closely related instance, where a translocation between chromosome No. 1 and 3 was present [18]

The following clinical case describes a patient whose clinical course fits unquestionably the clinical diagnosis of the so-called AAMM chromosomal mapping revealed the already described abnormality of chromosome No 8, which appears to be of an extremely ominous clinical significance and it can be considered a diagnostic marker of the acute with respect to the chronic form of the disease

Case Illustration

The patient was a 37-year-old woman whose family history was negative; however cousin (son of her father's sister) had died of acute leukemia. Parents, 7 brothers and 1 sister were alive and well

Past family history was unremarkable except for appendicectomy at age 17 and the excision of mammary nodul at age 34, which histologically



Fig. 1. Bone marrow biopsy showing extensive replacement of cell lines with monomorphous population of mononuclear cells. HE ×100.

Acute Agnogenic Myeloid Metaplasia with Chromosomal Abnormalities

Ettore Bartoli Giovannino Massarelli Giovanni Soggia Francesco Tanda and Maria Grazia Vianello

Istituto di Patologia Medica e Metodologia Clinica and Istituto di Anatomia e Istologia Patologica, Sassari University Sassari, and Istituto G Gaslini, Laboratorio di Citogenetica, Genova

Key Words. Acute myelofibrosis Chromosomal abnormalities Myeloproliferative disease Trisomy of chromosome No 8

Abstract. A case of a 37 year-old woman presenting with acute agnogenic myeloid metaplasia (AAMM) is described. The disease had a stormy course and was characterized by moderate splenomegaly persistently depressed WBC counts, extramedullary hemopoiesis and presence of a high percentage of atypical myeloblasts in the peripheral smear. Platelets were persistently low reticulocytes significantly below normal notwithstanding anemia. Hct tended to fall progressively to intolerably low values in the absence of transfusion

The chromosomal mapping of peripheral blood revealed the presence of a trisomy of chromosome No 8 This abnormality already demonstrated in two previous cases of acute myelofibrosis and the clinical course of the disease suggest that acute myelofibrosis and AAMM could be the same disease while chronic myelofibrosis should be considered a separate entity

Also it is possible that AAMM with trisomy of chromosome No 8 and stormy clinical course may be a different entity from the acute myeloproliferative disorders associated with other chromosomal abnormalities.

Acute agnogenic myeloid metaplasia is a relatively new entity Under this name are included patients affected by leukemia like syndromes similar to chronic myelofibrosis it differs from the latter by a lower degree of marrow fibrosis, the near absence of mega karyocytes, a more aggressive course the presence of abnormal myeloblasts in the peripheral smear and rapid transition into a rather malignant and invariably fatal form of acute leukemia, closely resembling erythroleukemia in most circumstances [1 3 7] Profound thrombocytopenia leading to frequent episodes of bleeding is a hallmark of the disease, which is also called acute myelofibrosis (AMF) or malignant myelosclerosis

The term acute agnogenic myeloid meta-

after all of them were 'dry taps' A bone marrow biopsy taken from the right posterior superior spina iliaca is shown in figure 1 the monomorphous cellular population substitutes normal marrow Silver impregnation (fig. 2) shows increased deposition of reticulum network. A liver biopsy was performed, which showed numerous foci of extramedullary hemopoiesis (fig. 3).

The patient bled after biopsy and the wound had to be closed surgically. Platelets were found to be 31,000/µl, PTT prolonged, the previous results were thought to be in error The peripheral smear showed consistently similar patterns. Myelofibrosis was diagnosed with the suspicion of AMF She was discharged on no therapy and readmitted 19 days later because of progressive anemia. Hct was 16%, Hb 4 7 g%, WCC 8,100- 1, 14 neutrophiles, 1 eosinophils, 40 lymphs, 4 myeloblasts, 1 myelocyte, 10 basophilic, 22 polychromatophilic and 8% orthochromatic normoblasts. Special cytochemical staining procedures performed on peripheral smears showed marked positivity for naphthol AS-D chloroacetate esterase.

Platelets were 45,000, reticulocytes 4,200/µl, serum iron 193 µg/dl (she had been transfused during the previous admission to repair bleeding) Fever supervened with daily spikes never exceeding 38 1 °C, but repeated blood and urine cultures were negative. Bone marrow aspiration was attempted again, yielding 'dry tap' bone biopsy was repeated and found unchanged. 10 days later peripheral blood smear was unchanged except for an increase in atypical blast forms up to 31 Menstrual bleeding was prominent and her menses were permanently halted with 0 25-mg estrogen–0 05-mg progesterone association, given by

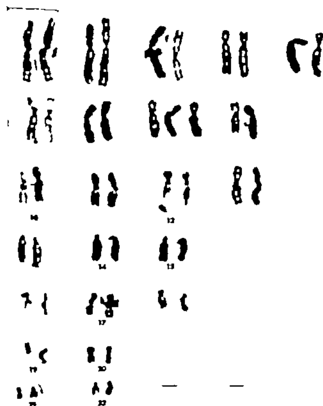


Fig. 4. Map showing trisomy of the 8th chromosome

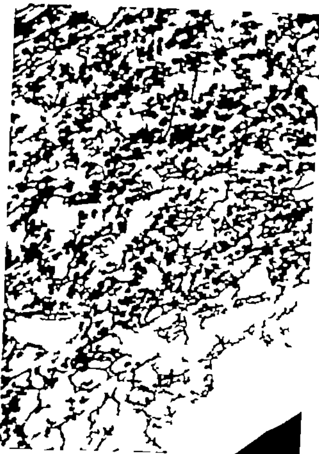


Fig. 2. Gomori's silver impregnation showing increased density and number of reticulin fibers. H. E. × 250

proved to be 'fibrous dysplasia of mammary gland with a voluminous distension cyst. Unmarried, she was gravida 1 para 1. Her daughter is alive and well. 3 months prior to admission the patient started complaining of mild fever with night peaks and sweats, accompanied by fatigue and abdominal pain referred to the left upper quadrant; she noticed that she was becoming progressively paler. She was referred to another hospital, where acute leukemia was diagnosed. the patient was transferred to our hospital. On admission, she appeared to be in no acute distress. She was pale, the spleen could be felt 2–3 cm below the left costal edge and was considerably hardened. Cervical lymph nodes were enlarged to the size of a pea. There were also a few enlarged lymph nodes on the right axilla. When questioned, she referred to prolonged bleeding during menses, and easy bleeding after teeth brushing and minor trauma. There were no additional abnormalities on physical examination. All routine laboratory tests were within normal limits, including HBsAg, except for an alkaline phosphatase of 42 mU/ml, SGOT 100 and SGPT 52 IU. Hct was 34%, Hb 11.5 g%, red cells 2.7 millions, white cells 6,500/μl, 43 neutrophiles, 1 eosinophile, 34 lymphocytes, 5 monocytes, 10 blast forms (thought to be myeloblasts), 5 erythroblasts, 1 myelocyte and 1 promyelocyte. Platelets were 110,000 μl, PT and PTT within normal limits, serum iron was 38 μg/dl. Ten bone marrow aspirations were attempted from different

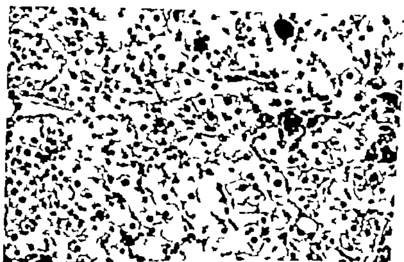


Fig. 3. Liver biopsy showing foci of extramedullary hemopoiesis within the si usoids. HE. × 250.

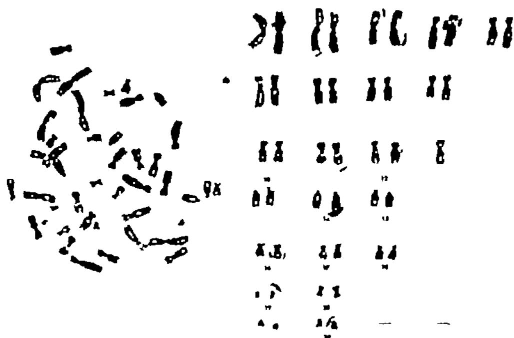


Fig. 5. Normal chromosomal map from bone marrow fibroblasts.

Discussion

AAMM is an uncertain entity recently recognized within the myeloproliferative syndromes [11]. Originally the denomination was warranted on clinical grounds only: it appeared that a small percentage of patients with myelofibrosis had a more aggressive disease and worse prognosis [1].

These patients had less splenomegaly, more frequent infection, and their peripheral blood smear contained (unlike chronic myelofibrosis) atypical myeloblasts in higher proportions than promyelocytes or metamyelocytes. This may be incorrectly diagnosed as chronic granulocytic leukemia if normoblasts present in peripheral blood are missed and bone marrow aspiration is not performed or fortuitously taken at a site not yet involved by the disease.

Recently a few cases of acute myelofibrosis were described whose clinical malignant course was suggestive of an acute type of leukemia, and whose genetics seemed to indicate that they might have represented a different entity rather than a variant of chronic myelofibrosis [12].

In patients with myelofibrosis and polycythemia vera clones of hemic cells bearing chromosomal abnormalities are relatively common, although they do not necessarily indicate progression of disease toward frank leukemia. Conversely in the other myeloproliferative disorders, including AMF and

mouth daily. She had episodes of retrobulbar and intraocular bleeding, petechial rashes and had to be transfused with packed red cells to keep her asymptomatic, which required a Hct above 15%. Platelets were given for counts lower than 15 000 μl and/or bleedings. No HL A matching was possible.

Chromosomal abnormalities were sought in peripheral blood cells and fibroblasts derived from bone biopsy. The blood sample was cultured in two different media, one containing RPMI medium, where incubation was protracted for 48 h in order to permit evaluation of spontaneous mitoses of certain myeloid origin. The other culture was protracted for 72 h in the presence of PHA in order to obtain the karyotype of normal cells. 48 h after beginning of culture 26 metaphases were recovered, these were almost invariably aneuploid because of gain or loss of one or more group C chromosomes. The clone encountered more frequently appears to be that containing an additional 8th chromosome, which was present in 9 metaphases (fig 4). In 8 metaphases, 5 of which had lost the X chromosome, the clone present contained only 45 chromosomes. On the contrary metaphases of cultures protracted for 72 h contained the normal female karyotype 46,XX. Bone biopsy specimen taken from the iliac crest yielded no cellular development, while rare clones grew from connective tissue cells, although with difficulties, all containing a normal karyotype. Over all, 79 cells were examined in the three culture mediums in no circumstance was it possible to evidence a structural aberration similar to those described by Van Slyck et al. [18]. The results are summarized in the table I. In figure 5 the materials examined are peripheral blood and bone biopsy. Peripheral blood cultures in the presence of PHA always produced normal metaphases, 46,XX. The same result was observed in all 12 metaphases obtained from fibroblasts derived by culture of bone biopsy; in addition, no structural abnormalities were seen. Peripheral blood elements incubated for 48 h in the absence of PHA demonstrated both nonmitotic disjunction and capacity to give rise to cellular clones. Among these, the most frequent one was that containing a supranumerary 8th chromosome which was observed in 9 out of 26 metaphases. Modal number of chromosomes was 46,XX/47,XX+8/45,XO. Thus, the diagnosis of AAMM was made based on the above clinical, histopathological, histochemical and chromosome findings.

The patient was discharged and readmitted 18 days later because of symptomatic anemia. Hct was 10%, Hb 4 g%, RBC 1.08 millions, platelets 160 000 μl, reticulocytes 0.2%, the peripheral smear showed that of 3,300 WBC, 15 were neutrophiles, 50 lymphs and 3% monocytes: remaining cells were proerythroblasts, atypical myeloblasts (11%), promyelocytes, myelocytes and metamyelocytes. All other laboratory examinations were normal, except for total proteins of 4.5 g dl, with 48% albumin, 5.7% α 11.7 α_2 16.7 β and 17.3 γ-globulins dl. LDH was invariably high (2,300 U/l). She was transfused but started bleeding with acute fall in platelet count following each transfusion, presumably due to antiplatelets antibody production. A trial with prednisone, 60 mg daily for 7 days, quickly tapered to a maintenance of 20 mg daily and of androstenolonum 50 mg day was without benefit. The patient was again discharged in poor conditions, to be followed in a chronic blood transfusion program. She died at home 3 weeks later of an acute massive bleeding episode. No autopsy could be performed.

Table I. Cytogenetic studies

Cell cultures	Number of metaphases	Karyotype
Bone biopsy (marrow)	–	
Bone biopsy (connective tissue fibroblasts)	12	46,XX
Peripheral blood	26	9 47,XX+8
(a) 48 h incubation without PHA (spontaneously dividing elements)		5 45,XO
		3 45,XX–C
		2 47,XX+9
		1 47,XX+11
		1 47,XX+12
		1 45,XX–21
		1 45,XX–20
		3 46,XX
(b) 72 h incubation with PHA (lymphocytes and small monocytes)	41	46,XX

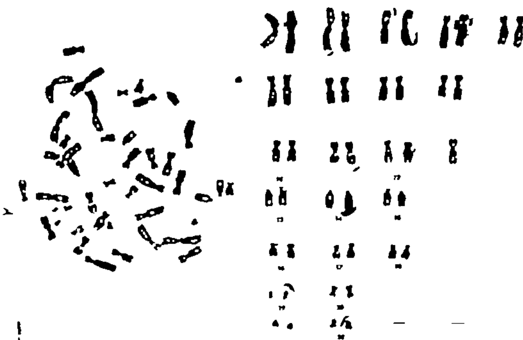


Fig. 5. Normal chromosomal map from bone marrow fibroblasts.

Discussion

AAMM is an uncertain entity recently recognized within the myeloproliferative syndromes [11]. Originally the denomination was warranted on clinical grounds only: it appeared that a small percentage of patients with myelofibrosis had a more aggressive disease and worse prognosis [1].

These patients had less splenomegaly, more frequent infection, and their peripheral blood smear contained (unlike chronic myelofibrosis) atypical myeloblasts in higher proportions than promyelocytes or metamyelocytes. This may be uncorrectly diagnosed as chronic granulocytic leukemia if normoblasts present in peripheral blood are missed and bone marrow aspiration is not performed or fortuitously taken at a site not yet involved by the disease.

Recently a few cases of acute myelofibrosis were described whose clinical malignant course was suggestive of an acute type of leukemia, and whose genetics seemed to indicate that they might have represented a different entity, rather than a variant of chronic myelofibrosis [1].

In patients with myelofibrosis and polycythemia vera clones of hemic cells bearing chromosomal abnormalities are relatively common, although they do not necessarily indicate progression of disease toward frank leukemia. Conversely in the other myeloproliferative disorders, including AMF and

pancytopenic dyscrasias, chromosomal abnormalities, when present, seem to bear a rather severe prognostic significance [11]

Cytogenetic studies were carried out in 2 cases of AMF evidencing an abnormal cellular clone that contained an extra chromosome belonging to the C group as shown by the banding technique. Trisomy of chromosome No 8 is the most common abnormality found in a variety of myeloid disorders, both preleukemic and leukemic, suggesting that acute myelofibrosis should be separated on clinical as well as cytogenetic grounds from the chronic form of the disease [10–14] An additional case of acute myelofibrosis previously studied showed that a 1–3 translocation had taken place, while chromosome No 8 was apparently uninvolved [18] For this reason additional individuals affected by this rare entity should be studied for the presence of trisomy of chromosome No 8

The case described in the present paper confirms these data. our patient had all the recognized typical features of AAMM Firstly splenomegaly was less than in chronic disease, although hard and painful The clinical course was stormy punctuated by frequent episodes of fever presumably reflecting infections because of extremely low absolute neutrophile counts, and of bleeding, with fatigue and symptoms very likely due to profound anemia. Bone marrow aspirates were always dry bone marrow biopsy was typical of acute myelofibrosis [9] Peripheral smear and peripheral blood leukocytes showed a few differential features. WBC counts never exceeded 10 000/μl, and a large variety of immature forms was always present, including atypical myeloblasts in a proportion larger than that of more mature myelocytic forms. Mature neutrophiles were invariably low and so were reticulocytes. The patient was incapable of maintaining a stable Hct and required repeated transfusions, which presumably resulted in formation of multiple antiplatelet antibodies.

Our patient was shown to bear within the peripheral blast forms the abnormal group C chromosome, an extra 8th chromosome like other patients reported with this clinical entity as previously pointed out by *Van Slyck et al.* [18] Bone marrow fibroblasts were successfully cultured yielding a normal karyotype. This finding again is suggestive of the secondary role played by fibroblast proliferation in AMF while the primary cell disturbance appears to reside exclusively within the hemopoietic cell lines.

In conclusion, we believe that the present case together with previous reports demonstrates that AAMM is a clinical variant of AMF and it is a true independent clinical entity In addition, this case strongly indicates that trisomy of chromosome No. 8 is an index that can be used to predict the clinical course of the disease. Alternatively it could be that AMF with trisomy of the chromosome No 8 and rapidly progressive disease could be a clinical entity distinct from the so-called AMF with other chromosomal abnormalities, such as that described by *Cehreli et al* [2] This requires, however studying and reporting additional cases investigated from a cytogenetic as well as clinical point of view

References

1 Bergsman, K. L. and Van Slyck, E. S. Acute myelofibrosis. An accelerated variant of agnogenic myeloid metaplasia. Ann. Intern. Med. *74* 232 235 (1971).

2 Cehreli, C., Erdinli, E. Z., Li, C. Y. and Krmpotic, E.: Blastic phase of agnogenic myeloid metaplasia simulating malignant lymphoma. Cancer 38: 1297–1305 (1976).

3 Evans, D. I. K.: Acute myelofibrosis in children with Down's syndrome. Archs Dis. Childh. 50: 458–462 (1975).

4 Gutierrez, A. C., Lisker, R. y Uribe, M.: Estudios citogeneticos en medula ósea en 185 pacientes con padecimientos hematológicos diversos. Rev. Invest. Clin., Mex. 26: 153–168 (1974).

5 Jensen, M. K. and Philip, P.: Cytogenetic studies in haematological disorders which may terminate in acute leukemia. Acta med. scand. 193: 353–387 (1973).

6 Kiossoglou, A. K., Mitus, W. J. and Dameshek, W.: Cytogenetic studies in the myeloproliferative syndrome. Blood 28: 241–247 (1966).

7 Lewis, S. M. and Szur, L.: Malignant myelosclerosis. Br. med. J. i: 472–477 (1963).

8 Linman, J. W. and Saarni, M. L.: The preleukemic syndrome. Semin. Hematol. 11: 93–100 (1974).

9 Lubin, J.; Rozen, S. and Rywlin, A. M.: Malignant myelosclerosis. Archs intern. Med. 136: 141–145 (1976).

10 Mitelman, F. and Levan, G.: Clustering of chromosome aberrations in specific chromosomes in human neoplasms. II. A survey of 287 cases. Hereditas 82: 167–174 (1976).

11 Mitus, W. J.; Coleman, N. and Kiossoglou, K. A.: Abnormal (marker) chromosomes in two patients with acute myelofibrosis. Archs intern. Med. 123: 192–197 (1969).

12 Nowell, P.; Jensen, J.; Gardner, F.; Murphy, S.; Chaganti, R. S. K., and German, J.: Chromosome studies in 'preleukemia. III. Myelofibrosis. Cancer 38: 1873–1881 (1976).

13 Nowell, P.: Marrow chromosome studies in 'preleukemia'. Further correlation with clinical course. Cancer 28: 513–518 (1971).

14 Philip, P.: Trisomy 8 in acute myeloid leukemia. Scand. J. Haematol. 14: 140–147 (1975).

15 Pierre, R. V.: Preleukemia states. Semin. Hematol. 11: 73–92 (1974).

16 Rowley, J. D.: Non-random chromosomal abnormalities in haematologic disorders of man. Proc. natn. Acad. Sci. USA 72: 152–156 (1975).

17 Silverstein, M. N., Brown, A. L., jr. and Linman, J. W.: Idiopathic myeloid metaplasia; its evolution into acute leukemia. Archs intern. Med. 132: 709–712 (1973).

18 Van Slyck, E. J.; Weiss, L., and Dully, M.: Chromosomal evidence for the secondary role of fibroblastic proliferation in acute myelofibrosis. Blood 36: 729–735 (1970).

19 Vianello, M. G.; Bonioli, E.: Importanza dell'identificazione delle bande Q e G nella citogenetica umana. Minerva Paediat. 26: 514–524 (1974).

20 Wurster-Hill, D., et al.: Chromosome studies in polycythemia vera. Semin. Hematol. 13: 13–32 (1976).

Received: March 6, 1979
Accepted: April 23, 1979

Prof. Ettore Bartoli,
Istituto di Patologia Medica,
Viale San Pietro 12, I-07100 Sassari (Italy)

Acta haemat. 62 214–218 (1979)

Chronic Myeloid Leukaemia and a Myasthenic Syndrome

Miriam A Wohl and J Keith Wood

Department of Haematology Leicester Royal Infirmary Leicester

Key Words. Myasthenic syndrome Eaton-Lambert syndrome Chronic myeloid leukaemia

Abstract. This is the first report of a case of chronic myeloid leukaemia (CML) complicated by myasthenic syndrome. The patient suffered two prolonged periods of idiosyncratic busulphan-induced marrow aplasia, the first occurring 5 months after busulphan was stopped. Between these two episodes, and at a time when no therapy was required for the leukaemia, myasthenic syndrome developed. After the patient recovered from the second episode, the myasthenic syndrome disappeared, and, despite subsequent recrudescence of CML, there were no further myasthenic symptoms, and treatment with neostigmine was discontinued.

Introduction

The myasthenic or Eaton-Lambert syndrome shows clinical and electrophysiological features which are in many respects different from those of myasthenia gravis [30] Cases have, however been reported with features of both [20 28]

The initial symptom is weakness and easy fatigability of the legs and/or less frequently the arms, both usually proximally Some patients may have blurring of vision or ptosis, although cranial muscle weakness is less prominent than in myasthenia gravis [22, 24] Tendon reflexes are almost always depressed or absent in contrast with the findings in myasthenia gravis. Although prolonged muscular activity produces fatigability a short period (10 sec) of voluntary muscular contraction (voluntary tetanus) may be followed by a brief increase in the maximal power of muscular contraction and by a transient return of the previously absent tendon reflex.

The typical dramatic response to cholinergic drugs seen in myasthenia gravis is variable in myasthenic syndrome, whereas guanidine hydrochloride, which has no convincing effect in myasthenia gravis, frequently produces an improvement [15 19 23] A marked sensitivity is seen among the patients to curare-like drugs [29] The ul-

trastructural appearances of the motor end plate differ from those in myasthenia gravis [1, 27] there is an increased area of post synaptic membrane which is decreased in myasthenia gravis [9]

Differentiation may be made by electromyography the amplitude of the muscle action potential evoked by stimulation of its nerve is markedly reduced (the decremental response) but is augmented with repetitive stimulation ('paradoxical potentiation or 'post-tetanic stimulation') [7 8] Examination of the thymus in patients dying with the myasthenic syndrome does not show histopathological changes of the type seen in myasthenia gravis [13]

An association between myasthenic syndrome and bronchial and other malignancies has been widely reported [2, 6, 7 13–17 23, 24] and it has also been described in the absence of neoplastic disease [–, 3, 10, 12, 21, 26] but myasthenia gravis has been found in connection with neoplasms (thymoma excepted) with only the same frequency that one would expect in the general population [25] Finally the pathogenic mechanisms of the two conditions differ (vide infra).

Case Report

In June 1973, 32-year-old male Kenyan Indian post-office worker presented at the Central Middlesex Hospital in London complaining of recurrent fevers and abdominal discomfort. Gross splenomegaly and white cell count of $623 \times 10^9/l$ were discovered. The blood and bone marrow films were diagnostic of classical chronic myeloid leukaemia, and treatment was begun with busulphan. This was interrupted when the white cell count was $20 \times 10^9/l$, and the condition remained under control without intervention for 5 months. Then marrow aplasia occurred with total white cell counts in the range $0.8–1.0 \times 10^9/l$. This phase was marked by 2 bouts of septicaemia, multiple transfusions, an ineffective course of oxymethalone and treatment with low dose of prednisolone, which, however had to be stopped because of gastrointestinal haemorrhage, probably precipitated by aspirin which the patient was taking surreptitiously for persistent fevers.

After he arrived in Leicester in May 1974, there was a slowly progressive recovery of bone marrow function, complicated by single episode of urinary tract infection and one of gastro-intestinal bleeding. Recurrent fevers defied elucidation, including comprehensive search for tuberculosis.

At the end of September 1974, he complained of muscular pain and weakness, particularly in the thighs and some difficulty in swallowing and speaking. Tendon reflexes were depressed but returned to normal after voluntary tetanus. Severe bilateral ptosis developed few days later and the diagnosis of myasthenic syndrome was confirmed by electromyography On tetanic stimulation, there was an initial decremental response, followed by the typical increase in potential amplitude (paradoxical potentiation). The effect of edrophonium (Tensilon) injection was as in myasthenia gravis, but after prolonged stimulation (4 min) some decremental response remained. It was not possible to test the effect of other drugs as the patient would not cooperate. Treatment with neostigmine was initially satisfactory but prednisolone 50 mg alt.die was later required to control weakness.

Reappearance of splenomegaly in December 1974 was accompanied by an increase in white cell count to $27 \times 10^9/l$ with normal platelet numbers. Busulphan therapy was cautiously recommenced t very low dose, only to be discontinued 8 weeks later because of thrombocytopenia.

In the early part of 1975, fevers were again prominent, and, in March, chest X-ray showed miliary tuberculosis, the diagnosis being confirmed by the findings of tubercle bacilli in urine, marrow and gastric washings. It seemed likely that tuberculosis was precipitated by steroid therapy and this was cautiously reduced and stopped in June 1975 Uneventful recovery from tuberculosis was secured with rifampicin, isoniazid and ethambutol.

Gastro-intestinal bleeding then recurred because of thrombocytopenia. Gastroscopy revealed the presence of severe haemorrhagic gastritis. A

laparotomy was carried out in July 1975 with splenectomy vagotomy and pyloroplasty The spleen was enlarged to six times the normal size and showed the expected histological features of chronic myeloid leukaemia. A few tubercles were removed from the abdomen, but no bacilli were identified on microscopy Following surgical intervention, gastro-intestinal bleeding ceased and bone marrow recovery began. A total of 140 U of blood and 30 U of platelet-rich plasma were needed in the 16 weeks prior to the laparotomy

5 months later gradual withdrawal of neostigmine was attempted, but myalgia resulted, and the drug was restarted. At no time, however was there a myasthenic crisis. The electromyograph of August 1976 was within normal limits, and neostigmine was then stopped with no untoward effect. He remains in haematological remission taking 6-thioguanine in August 1979

Discussion

This case has several atypical features

(1) It is unusual for idiosyncratic busulphan-induced marrow aplasia to occur 5 months after treatment is stopped. When busulphan was given for the second time, marrow depression appeared during treatment only a few weeks after it was started and this happened again on the third attempt, even at a very low dose.

(2) Myasthenic syndrome is uncommon in so young a patient, presumably because cancers tend to occur in an older age group

(3) There was a response to Tensilon, although this in no way invalidates the diagnosis [7 12] as the classical features of the syndrome were all present. (a) Tendon reflexes depressed after rest and increased after voluntary tetanus [19] (b) The electromyographic findings (vide supra) including paradoxical potentiation [7 8] (c) The presence of a neoplasm.

The concept of paraneoplastic syndromes is well documented, and, at any given time, 20% of a group of cancer patients in all stages of their diseases are suffering from a non-metastatic manifestation [11] There is an established association between myasthenic syndrome and malignancy classically intrathoracic [24] and usually oat cell, squamous, small-cell or anaplastic bronchogenic in type [2, 6 7 9 14 16, 17 23] Cases have also been reported with cancers of breast, prostate, stomach and rectum [13], non infiltrating papillary transitional cell carcinoma of bladder [15] reticulum cell sarcoma [24] and in patients suffering from Sjögren s syndrome [3] kidney stones [12], peripheral neuropathy [26] and thyrotoxicosis (report of 1 patient with benign prostatic hypertrophy a past history of excised basal cell carcinomas of the face and excised benign rectal polyps [21] There is one report of a case of hypothyroidism with pernicious anaemia [10] and several examples of patients in whom despite intensive investigation and follow up (some with post mortem findings included) no other pathological entity could be discovered [2, 12, 24]

The pathogenic mechanism of myasthenic syndrome is unknown. The patients have normal acetylcholine receptor (ACHR) content and no antibodies are present, but there is a defect in acetylcholine release [8] and an increase in total post-synaptic membrane area [18] Defective acetylcholine release may be due to factors caused by produced by or released by the neoplastic process. No such factors have yet been demonstrated. Since ACHR content is normal hypertrophy is probably confined to the membrane along the sides of the post-synaptic folds, which contain little or no acetylcholine receptors. The increase in membrane area may be a direct result of the factor(s) affect

ing acetylcholine release, rather than a trophic response of the muscle to reduced acetylcholine release by the nerve. Despite reports of cases with features of both disorders [20, 28] this contrasts sharply with classical myasthenia gravis, in which muscular weakness results from impaired neuromuscular transmission caused by reduced sensitivity of the post-synaptic membrane to acetylcholine (because antibodies cause loss of acetylcholine receptors [4 9 18] and in which there is a well-documented association with autoimmune disorders. We have found only three reports linking myasthenic syndrome to diseases with autoimmune concomitants [3 10, 21]

No satisfactory pathogenic mechanism has been established for myasthenic syndrome, nor do we propose one. The coexistence of chronic myeloid leukaemia and myasthenic syndrome may be coincidental, and we should be interested to hear of any similar experience, as myasthenic syndrome has not to date been reported in chronic myeloid leukaemia, or indeed in any other myeloproliferative disorder

Acknowledgement

W thank Dr *Marie Simpson* for electromyographic data and Dr *Paul Millac* for neurological advice.

References

1 Babakova, L. L.; Kolomenskaya, E. A., Ostrovskaya, N V and Pozdnyakov O. M. Investigation of the ultrastructure of neuromuscular synapses in the Lambert-Eaton myasthenic syndrome. Bull. Eksp. Biol. Med. *81* 69–80 (1976).

2 Brown, J C. and Johns, R. J Diagnostic difficulties encountered in the myasthenic syndrome sometimes associated with cancer J Neurol. Neurosurg. Psychiat. *37* 1214–1224 (1974).

3 Brown, J W.; Nelson, J R., and Herrmann, C., jr Sjögren's syndrome with myopathic and myasthenic features. Bull. Los Angeles Neurol. Soc. *33* 9–20 (1968).

4 Carnegie, P R. and Mackay I. R.. Vulnerability of cell-surface receptors to autoimmune reactions. Lancet *ii*. 684–686 (1975).

5 Davis, J N Myasthenia gravis. Br. J Hosp. Med. 2. 933–935 (1974).

6 Doi, H., Murai, Y and Kuroiwa, Y.: Eaton-Lambert syndrome, clinical report with special reference to facilitation of absent deep reflex. Rinsho Shinkeigaku *17* 593–599 (1977).

7 Eaton, L. M. and Lambert, E. H.. Electromyography and electric stimulation of nerves in diseases of motor unit: observations in myasthenic syndrome associated with malignant tumours. J Am. med. Ass. *163* 1117–1124 (1957).

8 Elmqvist, D and Lambert, E. H. Detailed analysis of neuromuscular transmission in patient with myasthenic syndrome sometimes associated with bronchogenic carcinoma. Mayo Clin. Proc. *43*. 689–713 (1968).

9 Fambrough, D. M.; Drachman, D. B., and Satyamurti, S.: Neuromuscular junction in myasthenia gravis; decreased acetylcholine receptors. Science *182*. 293–295 (1973).

10 Gutmann, L., Crosby T W Takamori, M., and Martin, J D.. The Eaton-Lambert syndrome and autoimmune disorders. Am. J Med. *53* 354–356 (1972).

11 Hall, T C. Paraneoplastic syndromes. Ann. N. Y Acad. Sci. *230*· 1 577 (1974).

12 Henriksson, K. G ; Nilsson, O., Rosén, I., and Schiller H.. Clinical, neurophysiological and morphological findings in Eaton-Lambert syndrome. Acta. neurol. *56*. 117–140 (1977).

13 Henson, R. A.. Neuromuscular disorders associated with malignant disease; in Walton, Disorders of voluntary muscle (Churchill/Livingstone, London 1974).

14 Kennedy W R. and Jimenez Pabon, E.. The myasthenic syndrome associated with small-cell carcinoma of the lung (Eaton-Lambert syndrome). Neurology *18*. 757–766 (1968).

15 Lambert, E. H.. Defects of neuromuscular transmission in syndromes other than myas-

thenia gravis. Ann. N Y Acad. Sci. *135* 367–384 (1966).

16 Lambert, E. H. and Rooke, E. D., Myasthenic state and lung cancer in Brain and Norris, The remote effects of cancer on the nervous system (Grune & Stratton, New York 1965).

17 Lambert, E. H. Rooke, E. D Eaton, L. M., and Hodgson, C. H., in Viets, Myasthenia gravis (Thomas, Springfield 1961).

18 Lindstrom, J M. and Lambert, E. H., Content of acetylcholine receptors and antibodies bound to receptors in myasthenia gravis, autoimmune myasthenia gravis and Eaton-Lambert syndrome. Neurology *28* 130–138 (1978)

19 McQuillen, M. P and Johns, R. J., The nature of the defect in the Eaton Lambert syndrome. Neurology Minneap *17* 527–536 (1967).

20 Mori, M. and Takamori, M. Hyperthyroidism and myasthenia gravis with features of Eaton-Lambert syndrome. Neurology *26*. 882–887 (1976)

21 Norris, F H., jr., Neuromuscular transmission in thyroid disease. Ann. intern. Med. *64* 81–86 (1966).

22 Oh, S. T., The Eaton-Lambert syndrome in ocular myasthenia gravis. Archs Neurol., Chicago *31* 183–186 (1974).

23 Oh, S. T and Kim, K. W., Guanidine hydrochloride in the Eaton-Lambert syndrome. Neurology Minneap. *23* 1084–1090 (1973).

24 Rooke, E. D Eaton, L. M., Lambert, E. H., and Hodgson, C. H., Myasthenia and malignant intrathoracic tumor. Med. Clins N. Am. *44* 977–988 (1960).

25 Rowland, L. P and Schotland, D. L. Neoplasms and muscle disease, in Brain and Norris, Remote effects of cancer on the nervous system (Grune & Stratton, New York 1965).

26 Ryan, D W and Cherington, M. Human type A botulism. J Am. med. Ass. *216*. 513–514 (1971)

27 Santa, T., Engel, A. S., and Lambert, E. H. Histometric study of neuromuscular junction ultrastructure in myasthenic syndrome. Neurology Minneap. *22*. 370–376 (1972).

28 Schwartz, M. S. and Stålberg, E., Myasthenia gravis with features of the myasthenic syndrome. Neurology Minneap. *25* 80–84 (1975).

29 Tyler H. R., Paraneoplastic syndromes of nerve, muscle and neuromuscular junction. Ann. N Y Acad. Sci. *230* 348–357 (1974).

30 Walton, J N., Brain s diseases of the nervous system, 8th ed. (Oxford University Press, London 1977).

Received. September 15, 1977
Accepted. April 27 1979

Miriam A. Wohl, Department of Haematology
The Leicester Royal Infirmary
Leicester LE1 5WW (England)

Acta haemat. 62: 219–222 (1979)

Elevated Plasma β-Thromboglobulin Levels in Multiple Myeloma and in Polycythaemia vera

Guntram Schernthaner, Heinz Ludwig and Karl Silberbauer[1]

Department of Internal Medicine II, University of Vienna, Vienna

Key Words. β-Thromboglobulin · Multiple myeloma · Polycythaemia vera · Thrombohaemorrhagic complications

Abstract. Plasma β-thromboglobulin levels were determined in 22 multiple myeloma patients, in 15 patients with polycythaemia vera and in 70 healthy controls. In both multiple myeloma and polycythaemia vera significantly increased plasma concentrations of this platelet-specific protein were found.

Recently β-thromboglobulin (β-TG) has been defined by *Moore et al.* [12]. This platelet-specific β-globulin is released specifically when platelets aggregate. In some disorders with increased incidence of coagulopathic complications, as for example in deep venous thrombosis [10], pre-eclampsia [17], myocardial infarction [7], and also in a considerable number of diabetes mellitus cases [16, 19] elevated β-TG levels have been reported up to now.

In patients with multiple myeloma or polycythaemia vera thrombohaemorrhagic complications are well known [4, 14, 20, 22]. Among the factors responsible for this phenomenon, disturbed intravascular platelet aggregation might play a major role. Thus, it was of interest to determine levels of plasma β-TG as an indicator for disturbed platelet aggregation in patients with multiple myeloma and polycythaemia vera.

The authors would like to thank Mrs *E. Freisinger* for the excellent technical assistance.

Patients and Methods

22 multiple myeloma patients and 15 patients with polycythaemia vera were studied. Normal control samples were obtained from 70 healthy individuals.

Plasma β-TG was determined by radioimmunoassay with kits from the Radiochemical Centre, Amersham. Blood samples were obtained by clean venopuncture of an antecubital vein and prepared carefully for determination of plasma β-TG as described by *Bolton et al.* [2]. None of the probands had taken antiplatelet drugs during 14 days prior to testing.

thenia gravis. Ann. N Y Acad. Sci. *135* 367–384 (1966).
16 Lambert, E. H. and Rooke, E. D., Myasthenic state and lung cancer in Brain and Norris, The remote effects of cancer on the nervous system (Grune & Stratton, New York 1965).
17 Lambert, E. H. Rooke, E. D Eaton, L. M., and Hodgson, C. H. in Viets, Myasthenia gravis (Thomas, Springfield 1961).
18 Lindstrom, J M. and Lambert, E. H., Content of acetylcholine receptors and antibodies bound to receptors in myasthenia gravis, autoimmune myasthenia gravis and Eaton-Lambert syndrome. Neurology *28* 130–138 (1978)
19 McQuillen, M. P and Johns, R. J The nature of the defect in the Eaton-Lambert syndrome. *Neurology Minneap. 17 527–536 (1967)*
20 Mori, M. and Takamori, M., Hyperthyroidism and myasthenia gravis with features of Eaton-Lambert syndrome. Neurology 26. 882–887 (1976)
21 Norris, F H., jr Neuromuscular transmission in thyroid disease. Ann. intern. Med. *64* 81–86 (1966).
22 Oh, S. T., The Eaton-Lambert syndrome in ocular myasthenia gravis. Archs Neurol., Chicago *31* 183–186 (1974)
23 Oh, S. T and Kim, K. W., Guanidine hydrochloride in the Eaton Lambert syndrome. Neurology Minneap. *23* 1084–1090 (1973)
24 Rooke, E. D., Eaton, L. M., Lambert, E. H., and Hodgson, C. H., Myasthenia and malignant intrathoracic tumor Med. Clins N. Am. *44* 977–988 (1960).
25 Rowland, L. P and Schotland, D L. Neoplasms and muscle disease, in Brain and Norris, Remote effects of cancer on the nervous system (Grune & Stratton, New York 1965).
26 Ryan, D W and Cherington, M. Human type A botulism. J Am. med. Ass. *216.* 513–514 (1971).
27 Santa, T Engel, A. S., and Lambert, E. H. Histometric study of neuromuscular junction ultrastructure in myasthenic syndrome. Neurology Minneap. *22.* 370–376 (1972).
28 Schwartz, M. S. and Stålberg, E. Myasthenia gravis with features of the myasthenic syndrome. Neurology Minneap. *25* 80–84 (1975).
29 Tyler H. R. Paraneoplastic syndromes of nerve, muscle and neuromuscular junction. Ann. N Y Acad. Sci. *230.* 348–357 (1974).
30 Walton, J N., Brain's diseases of the nervous system, 8th ed. (Oxford University Press, London 1977).

Received. September 15, 1977
Accepted. April 27 1979

Miriam A. Wohl, Department of Haematology
The Leicester Royal Infirmary
Leicester LE1 5WW (England)

trations have been published to the best of our knowledge up to now In this disorder several abnormalities of platelet function [8, 18], especially decreased adhesiveness, impaired platelet aggregation and reduced availability of platelet factor 3 [13] have been documented. Thus, the present findings of increased β-TG concentrations in multiple myeloma seem to be of particular interest, since this observation is likely to indicate increased *in vivo* and/or *in vitro* platelet activity However it can not be excluded at present that in this disorder elevated β-TG levels are only the reflection of particularly leaky platelets.

In conclusion, the above data show significantly increased β-TG plasma concentrations in patients with multiple myeloma and polycythaemia vera. However since very little is known about the function or regulators of production and release of this platelet product, further follow-up studies seem essential in order to clarify the clinical importance of our findings.

References

1 Berger S Aledort, L. M., Gilbert, H. S., Hanson, J P and Wasserman, L. R. Abnormalities of platelet function in patients with polycythemia vera. Cancer Res 33. 1683–1687 (1973)

2 Bolton, A. E., Ludlam, C. A.; Moore, S Pepper D. S., and Cash, J D. Three approaches to the radioimmunoassay of human β-thromboglobulin. Br J Haemat. 33 233–238 (1976).

3 Boughton, B J Allington, M. J and King, A. Platelet and plasma β-thromboglobulin in myeloproliferative syndromes and secondary thrombocytosis. Br J Haemat. 40 125–132 (1978).

4 Catovsky D Ikoku, N. B., Pitney W R., and Galton, D A. G Thromboembolic complications in myelomatosis. Br med. J III. 438–439 (1970).

5 Cooper, B., Schafer A. J Pachalsky D., and Handin, R. L. Platelet resistance to prostaglandin D_2 in patients with myeloproliferative disorders. Blood 52. 628–636 (1978).

6 Dawson, A. A. and Ogston, D. The influence of platelet count on the incidence of thrombotic and haemorrhagic complications in polycythaemia vera. Post-grad. med. J 46. 76–78 (1970).

7 Denham, M. J Fisher M., James, G., and Hansen, M. β-Thromboglobulin and heparin-neutralising activity test in clinical conditions. Lancet I. 1154 (1977).

8 Kasturi, J and Saraya, A. K. Platelet functions in dysproteinaemia. Acta haemat. 59 104–113 (1978).

9 Keenan, J P.; Wharton, J Shepherd, A. J N and Bellingham, A. J Defective platelet lipid peroxidation in myeloproliferative disorders. A possible defect of prostaglandin synthesis. Br J Haemat. 35. 275–283 (1977).

10 Ludlam, C. A., Moore, S., Bolton, A. E., and Cash, J D New rapid method for diagnosis of deep venous thrombosis. Lancet II. 259–260 (1975).

11 Maldonado, J E., Pintado, T and Pierre, R. V Dysplastic platelets and circulating megakaryocytes in chronic myeloproliferative diseases. I. The platelets: ultrastructure and peroxidase reaction. Blood 43 797–809 (1974).

12 Moore, S.; Pepper, D. S., and Cash, J D The isolation and characterisation of platelet-specific β-globulin (β-thromboglobulin) and the detection of anti-urokinase and antiplasmin released from thrombin-aggregated washed human platelets. Biochim. Biophys. Acta 379 360–369 (1975).

13 Pachter M. R., Johnson, S. A., and Basinski, D. H. The effect of macroglobulins and their dissociation units on release of platelet factor 3. Thromb. Diath. haemorrh. 3. 501 509 (1959).

14 Pearson, T C. and Wetherley-Mein, G Vascular occlusive episodes and venous haematocrit in primary proliferative polycythaemia. Lancet II 1219–1222 (1978).

15 Perkins, H. A., Mackenzie, M. R., and Fudenberg, H. H. Hemostatic defects in dysproteinemias. Blood 35 695–707 (1970).

16 Preston, F E., Ward, J D.; Marcola, B. H.;

Results and Discussion

The mean concentration of plasma β-TG was significantly higher in the group of patients with polycythaemia vera ($p < 0.01$) or multiple myeloma ($p < 0.001$) than in normal subjects (fig. 1). The values obtained in the controls varied from 8 to 60 ng/ml and showed only minor variations in 10 control subjects in whom β-TG concentrations were determined repeatedly during 3 consecutive days.

11 of the 22 multiple myeloma patients and 12 of the 15 patients with polycythaemia vera showed plasma β-TG levels distinctly higher than normals. There was no significant correlation between β-TG concentrations and platelet count number. Likewise no correlation between thromboembolic complications in the patients studied and β-TG levels was found.

The finding of significantly increased β-TG levels in patients with polycythaemia vera confirms the recently published data obtained in 5 patients with this disorder by *Boughton et al.* [3]. Reported platelet abnormalities in patients with myeloproliferative disorders include changes in the number of platelets, the presence of circulating platelet aggregates, and abnormalities in platelet morphology and granule contents as well as defective aggregation, release reaction, and lipid peroxydation [1, 6, 9, 11, 15, 21, 22]. In addition in patients with myeloproliferative disorders, platelet resistance to prostaglandin D2 was reported recently [5].

In patients with multiple myeloma no systematic studies evaluating β-TG concen-

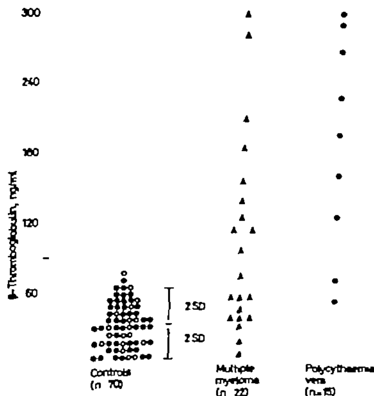


Fig. 1. Plasma β-TG concentrations in patients with multiple myeloma (MM) or polycythaemia vera (PV) in comparison to healthy controls (C). MM vs. C: $p < 0.01$; PV vs. C: $p < 0.001$ (Student's t test after log transformation).

Acta haemat. 62, 223–228 (1979)

Thrombocytosis, Thrombocythaemia and Iron Deficiency in Patients with Polycythaemia vera

D. Shaw and G. R. Tudhope

Department of Pharmacology and Therapeutics, University of Dundee, Ninewells Hospital, Dundee

Key Words. Iron deficiency · Polycythaemia vera · Thrombocythaemia · Thrombocytosis

Abstract. 6 patients with polycythaemia vera who also developed concomitant iron deficiency are reported. When oral iron therapy was given, there was a marked reduction in the previously elevated platelet counts in 4 patients: during 2–9 years of observation of these patients there was a significant inverse relationship between haemoglobin concentration and platelet count. In the other 2 patients the elevated platelet counts did not fall when iron therapy was given. The significance of these different patterns of response in relation to the causal mechanism of elevated platelet counts in patients with polycythaemia vera is discussed.

Introduction

Patients with iron deficiency anaemia may have decreased, normal or moderately elevated peripheral blood platelet counts [8, 16]. It has been shown in patients who have iron deficiency anaemia associated with moderate thrombocytosis that the platelet count returns to the normal range when the iron deficiency is corrected [8, 16]. In polycythaemia vera the myeloproliferative process commonly involves the megakaryocyte and leukocyte series as well as the erythrocytes and over 60% of patients with this disease have a platelet count in excess of 400×10^9/l at the time of initial diagnosis [18].

We present evidence to suggest that elevated platelet counts in polycythaemia vera are not always due to the myeloproliferative process. In some patients the response in the platelet count to iron therapy is similar to that which occurs in patients with iron deficiency from various causes.

Patients and Methods

6 patients with polycythaemia vera who were treated for concomitant iron deficiency were studied. The red cell and plasma volumes were measured by the ^{51}Cr-labelled autologous red cell dilution method and ^{125}I-labelled albumen dilution method respectively [10]. Haematological investigations including marrow examination were

Porter N R., Timperley W R., and O Malley B C., Elevated β-thromboglobulin levels and circulating platelet aggregates in diabetics microangiopathy Lancet *I*, 238–239 (1978).
17 Redman, C. W G., Allington, M. J., Bolton, F G and Stirrat, G M., Plasma β-thromboglobulin in preeclampsia. Lancet *II*, 248 (1977)
18 Saraya, A. K. Kasturi, J and Kishan, R., A study of haemostasis in macroglobulinaemia. Acta haemat. *47* 33–42 (1972)
19 Schernthaner G Silberbauer K. F Sinzinger H., Pirz Katzer M. und Winter M., Verminderte vaskuläre Prostacyclin-(PG I_2)-Synthese und erhöhte Plasma-β-Thromboglobulinkonzentration beim Diabetes mellitus. Verh. dt. Ges. inn. Med. (im Druck).
20 Wasserman, L. R. and Gilbert, H. S., Complications of polycythemia vera. Semin. Hematol. *3* 199–208 (1966).
21 Weinfeld, A., Branehög, L, and Kubbl, J Platelets in the myeloproliferative syndrome. Clin. Haematol. *4* 373 (1975).
22 Wu, K. K., Platelet hyperaggregability and thrombosis in patients with thrombocythemia. Ann. intern. Med. *88* 7–11 (1978).

Received. February 28, 1979
Accepted. April 23, 1979

G Schernthaner MD
II. Medizinische Universitätsklinik,
Garnisongasse 13, A–1090 Wien IX (Austria)

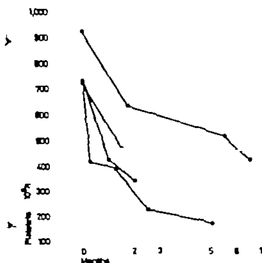


Fig. 1. Platelet counts in 4 cases of polycythaemia vera and concurrent hypoferraemia during treatment with oral iron.

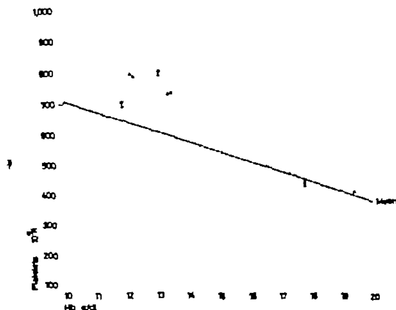


Fig. 2. Platelet counts and haemoglobin measurements in 4 cases of polycythaemia vera and concurrent hypoferraemia.

performed using standard methods. The normal range for the peripheral blood platelet count was taken as 150–400 $\times 10^9$/l [3]. Iron deficiency was diagnosed when the serum iron was 7.5 μmol/l or less and the concomitant total iron binding capacity was in the upper part of the normal range or greater (normal range 36–77 μmol/l). Secondary polycythaemia was excluded by history, clinical examination, chest X-ray, intravenous pyelography and, when the possibility of pulmonary disease was considered, lung function tests. Treatment was by venesection or intravenous injections of 3–5 mCi of radiophosphorus or both. Iron therapy was given as ferrous sulphate tablets, 200 mg three times per day.

Results

The haematological data at the time of presentation are shown in table I. Bone marrow examination was carried out on 4 patients. On microscopy there was general hypercellularity with considerable reduction in the amount of fat. In cases 1, 2 and 3 the increase in number of megakaryocytes was particularly marked. The marrow findings were consistent with a diagnosis of myeloproliferative disease.

2 patients (cases 1 and 2) showed evidence of iron deficiency at the time of initial diagnosis. In case 1 fecal occult blood tests were positive and barium enema showed multiple diverticulae; the platelet count was 800 $\times 10^9$/l, but the haemoglobin was normal initially (table I) and the polycythaemia did not become apparent until the iron deficiency was corrected. In case 2 the hypoferraemia at the time of diagnosis was probably due to previous bleeding from external haemorrhoids; treatment with radiophosphorus was given following a course of oral iron. In cases 3–6 therapy of polycythaemia had been given before iron deficiency developed. Cases 3 and 5 had been treated by venesections alone and cases 4 and 6 by venesections and radiophosphorus. Each of these 4 received a course of oral iron on one occasion when they were found to be hypoferraemic; there was an interval of at least 3 months between the last therapeutic venesection and the start of iron therapy. None was known to be bleeding.

4 (cases 1, 3, 4 and 5) of the 6 patients showed a marked reduction in the peripheral blood platelets following iron therapy (fig. 1). The initial platelet results were confirmed by counts on two or more samples. During iron therapy the haemoglobin levels

Table I. Haematological data at the time of presentation in 6 patients with polycythaemia vera

	Patient					
	1	2	3	4	5	6
Age, years	69	62	67	41	67	61
Sex	F	F	F	M	M	F
Hb, g/dl	13.1	14.9	19.2	19.6	17.1	21.6
Hct	0.45	0.48	0.63	0.62	0.54	–
WBC, $\times 10^9$/l	16.2	22.7	32.2	22.9	8.8	–
Platelets, $\times 10^9$/l	800	850	350	935	630	1,250
RBC vol, ml/kg	40.9	34.3	46.0	52.0	46.3	43.9
Spleen, cm below costal margin	5	2	0	0	2	0
Marrow aspirate	+	+	+	+	–	–

is associated with a disorder of the bone marrow which has many of the characteristics of a malignant disease. There has been much interest in the heterogeneity in size of megakaryocytes and platelets [13] The mean platelet diameter was found to be considerably increased in polycythaemia vera [18] and the enlarged megakaryocytes found in thrombocythaemia contrast with the decrease in megakaryocyte size in reactive thrombocytosis [9] Observations of platelet and megakaryocyte size were not made in the present cases. However in 4 of the patients in this study who were also hypoferraemic there was a rapid fall in the platelet counts when they were given oral iron. This response would not be expected if the excessive number of platelets in the peripheral blood had been due to autonomous production by malignant megakaryocytes. It appears that, in these patients, the elevated counts reflected a thrombocytosis resulting from iron deficiency and not thrombocythaemia.

There appears to be only one previous published report on polycythaemia vera when iron deficiency was associated with an abnormal platelet count [15] In that single case thrombocytopenia was present and was corrected by oral iron. In contrast, in the 4 patients in the present study a different mechanism was affected and it appears that the inhibitory effect of iron was lost during iron deficiency

2 patients who were iron deficient did not show a reduction in the platelet count when given oral iron. Any inhibitory effect of iron was not seen in these particular patients and it must be assumed that this was due to the autonomy of the megakaryocytes and that the elevated platelet counts were a true thrombocythaemia.

These findings indicate that patients who present with an elevated platelet count and a low or normal haemoglobin should be investigated for iron deficiency and the possible diagnosis of polycythaemia vera considered. Further in patients with polycythaemia vera any elevated platelet count should not necessarily be considered to be a thrombocythaemia without a knowledge of the iron status.

Acknowledgements

W are grateful to Mr *S. A. Ogston* for statistical advice.

References

1 Beard, M. E. J and Johnson, S. A. N Thrombocytopenia and iron deficiency anaemia in patient with ,-thalassaemia trait. Response to iron therapy Acta. haemat. 59 114–118 (1978).

2 Choi, S. and Simone, J V Platelet production in experimental iron deficiency anaemia. Blood 42. 219–228 (1973).

3 Dacie, J V and Lewis, S. M.: Practical haematology; 4th ed., p.74 (Churchill, London 1968).

4 Dincol, K. and Aksoy M. On the platelet levels in chronic iron deficiency anaemia. Acta haemat. 41 135–143 (1969).

5 Dincol, K. and Aksoy M.: Iron deficiency and thrombocytosis. Archs Intern. Med. 133 1070–1070 (1974).

6 Freedman, M. L. and Karpatkin, S.. Requirement of iron for platelet protein synthesis. Biochem. biophys. Res. Commun. 54 475–481 (1973).

7 Garg, S. K., Weiner, M., and Karpatkin, S. Thrombocyte and megathrombocyte kinetics during thrombocytosis induced by acute and chronic blood loss and by iron-deficiency diet. Haemostasis 1: 121–135 (1972/73).

8 Gross, S; Keefer V and Newman, A. J The platelets in iron-deficiency anaemia. I. The re-

Table II. Haemoglobin concentrations before and after oral iron therapy in 6 patients with polycythaemia vera and concurrent hypoferraemia

Case No.	Haemoglobin concentration, g/dl		
	before iron therapy	after iron therapy	duration of iron therapy months
1	12.3	20.0	5
2	14.9	16.5	1
3	13.5	15.3	6
4	11 7	19 4	2
5	10.4	20.4	6
6	14.3	16.4	4

rose steadily and the initial and final levels are shown in table II. To assess whether or not there was an overall inverse relationship between the platelet count and the haemoglobin level in these 4 patients with concurrent hypoferraemia, all the haemoglobin levels for the 2–9 years that these patients have been treated have been plotted against the platelet counts (fig. 2). When the patients had low haemoglobin levels they were shown to be hypoferraemic by serum iron and total iron binding capacity assays. The mean regression line is shown in the graph and the slope is statistically significant ($r = -0.49$ $p < 0.001$) In the other 2 cases, the platelet counts did not fall significantly when iron therapy was given. Neither patient was known to be bleeding at the time.

Discussion

Iron deficiency anaemia may be associated with a normal or an increased platelet count and less frequently in adults, a reduced count [1 8, 12, 14 16 17] *Dincol and Aksoy* [4 5] have presented evidence that the thrombocytosis associated with iron deficiency is due to active blood loss. However it has been confirmed in animal experiments that dietary deficiency of iron without concomitant blood loss can result in thrombocytosis [2, 7 11 16]

Karpatkin et al. [11] have suggested two mechanisms by which iron influences the platelet count. Firstly iron, directly or indirectly inhibits the rise in the peripheral blood platelet count, above the normal range, possibly via thrombopoietin ('inhibitory compartment') they postulate that the thrombocytosis of iron deficiency results from failure of this inhibition. Secondly on the basis of their observations that iron is required for platelet protein synthesis [6] and, separately for maximal thrombopoiesis [11] they postulated that iron influences the platelet count via other metabolic pathways ('essential components compartment') and that iron deficiency acting on these pathways, tends to produce thrombocytopenia. According to this hypothesis, depending upon which of these two mechanisms is more affected, iron deficiency can give rise to either an increase or decrease in the platelet count. Whether the difference between observed thrombocytosis in some patients and thrombocytopenia in others is simply a difference in the degree of iron deficiency is not known.

The patients in this study differ from those in the above studies in that they had polycythaemia vera and in this disease the platelet count is above the normal range at the time of diagnosis in over 60% of patients [18] An excess of platelets in a patient with a myeloproliferative disease is generally considered to be a thrombocythaemia, as distinct from a thrombocytosis, as it

Acta haemat. 62: 229–235 (1979)

Anti Lymphocyte Antibody Levels in Chronic Lymphocytic Leukaemia

C. M. Lewis and G. D. Pegrum

Haematology Department, Charing Cross Hospital Medical School, London

Key Words.

Abstract. A radioimmunoassay for measuring levels of lymphocyte autoantibody in chronic lymphocytic leukaemia (CLL) has been developed. Antibody in the form of crude IgG was extracted from patients' sera and iodinated. The assay utilizes its cross-reactivity with other CLL cells. Levels were measured in 23 patients. The results show that an inverse relationship exists between the quantity of circulating CLL autoantibodies and the number of mouse red blood cell rosetting lymphocytes (M cells). The preliminary findings do not correlate with disease activity although it is our impression that patients who are maintaining higher levels of autoantibody and fewer M-rosetting cells have non-progressive disease.

Introduction

Although the humoral immune response is considered to be impaired in chronic lymphocytic leukaemia (CLL), autoimmune antibodies with specificity for red cells and platelets are likely to occur [3, 4] and it has also been noted that cytotoxic antibodies to the leukaemic cells may develop [10]. We have found that antilymphocyte antibodies present in sera from these patients affect the behaviour of the CLL cells and that these antibodies cross-react extensively from patient to patient [9]. Their biological activity can be demonstrated *in vitro* through the enhancement of leukaemic cell viability and this suggested that lymphocyte antibodies may be responsible for longevity of CLL cells *in vivo*.

The extensive cross-reactivity of sera from these patients supports the idea of a common leukaemic antigen [11], but more important it was considered that this could be used to develop a radioimmunoassay. This would enable us to quantify the amount of antileukaemic antibodies and determine whether the level in different patients bears any relationship to their clinical condition or other parameters. This paper describes the method we developed and the results found.

sponse to oral and parenteral iron. Pediatrics *34* 315–323 (1964)

9 Harker L. A., The kinetics of platelet production and destruction in man. Clin. Haematol. *6*. 671–693 (1977).

10 International Committee for Standardization in Haematology Br J Haemat. *25* 801–814 (1973)

11 Karpatkin, S Garg, S. K., and Freedman, M. L.. Role of iron as a regulator of thrombopoiesis. Am. J Med. *57* 521–525 (1974).

12 Knizley H. and Noyes, W D Iron deficiency anaemia, papilloedema, thrombocytosis and transient hemiparesis. Archs intern. Med. *129* 483–486 (1972)

13 Penington, D G Streatfield, K., and Roxburgh, A. E.. Megakaryocytes and the heterogeneity of circulating platelets. Br J Haemat. *34* 639–652 (1976)

14 Sahud, M. A., Olsen, H., and Pedemont, L.. Thrombocytopenia and iron deficiency Ann. intern. Med. *81* 132–133 (1974).

15 Scher H. and Silber R. Iron responsive thrombocytopenia. Ann. intern. Med. *84* 571–572 (1976).

16 Schloesser L. L., Kipp, M. A., and Wenzel, F J Thrombocytosis in iron deficiency anaemia. J Lab. clin. Med. *66* 107–114 (1965).

17 Sonneborn, D.. Thrombocytopenia and iron deficiency Ann. intern. Med. *80* 111 (1974).

18 Weinfeld, A., Branehog, I., and Kutti, J Platelets in the myeloproliferative syndrome. Clin. Haematol. *4* 373–392 (1975).

Received. *March 27 1979*
Accepted. *May 10, 1979*

Dr D Shaw Department of
Pharmacology and Therapeutics,
University of Dundee, Ninewells Hospital,
Dundee DD2 1UB (Scotland)

Acta haemat. 62: 229–235 (1979)

Anti-Lymphocyte Antibody Levels in Chronic Lymphocytic Leukaemia

C.M. Lewis and G.D. Pegrum

Haematology Department, Charing Cross Hospital Medical School, London

Key Words.

Abstract. A radioimmunoassay for measuring levels of lymphocyte autoantibody in chronic lymphocytic leukaemia (CLL) has been developed. Antibody in the form of crude IgG was extracted from patients' sera and iodinated. The assay utilizes its cross-reactivity with other CLL cells. Levels were measured in 23 patients. The results show that an inverse relationship exists between the quantity of circulating CLL autoantibodies and the number of mouse red blood cell rosetting lymphocytes (M cells). The preliminary findings do not correlate with disease activity although it is our impression that patients who are maintaining higher levels of autoantibody and fewer M rosetting cells have non-progressive disease.

Introduction

Although the humoral immune response is considered to be impaired in chronic lymphocytic leukaemia (CLL) autoimmune antibodies with specificity for red cells and platelets are likely to occur [3, 4] and it has also been noted that cytotoxic antibodies to the leukaemic cells may develop [10]. We have found that antilymphocyte antibodies present in sera from these patients affect the behaviour of the CLL cells and that these antibodies cross-react extensively from patient to patient [9]. Their biological activity can be demonstrated *in vitro* through the enhancement of leukaemic cell viability and this suggested that lymphocyte antibodies may be responsible for longevity of CLL cells *in vivo*.

The extensive cross-reactivity of sera from these patients supports the idea of a common leukaemic antigen [11], but more important it was considered that this could be used to develop a radioimmunoassay. This would enable us to quantify the amount of antileukaemic antibodies and determine whether the level in different patients bears any relationship to their clinical condition or other parameters. This paper describes the method we developed and the results found

sponse to oral and parenteral iron. Pediatrics *34* 315–323 (1964).

9 Harker L. A. The kinetics of platelet production and destruction in man. Clin. Haematol. *6* 671–693 (1977)

10 International Committee for Standardization in Haematology Br J Haemat. *25* 801–814 (1973)

11 Karpatkin, S., Garg, S. K., and Freedman, M L. Role of iron as a regulator of thrombopoiesis. Am. J Med. *57* 521–525 (1974)

12 Knizley H. and Noyes, W D Iron deficiency anaemia, papilloedema, thrombocytosis and transient hemiparesis. Archs intern. Med. *129* 483–486 (1972).

13 Penington, D G., Streatfield, K., and Roxburgh, A. E. Megakaryocytes and the heterogeneity of circulating platelets. Br J Haemat. *34* 639–652 (1976)

14 Sahud, M. A., Olsen, H., and Pedemont, L. Thrombocytopenia and iron deficiency Ann. intern. Med. *81* 132–133 (1974).

15 Scher H. and Silber R. Iron responsive thrombocytopenia. Ann. intern. Med. *85* 571–572 (1976)

16 Schloesser L. L., Kipp, M. A., and Wenzel, F J Thrombocytosis in iron deficiency anaemia. J Lab clin. Med. *66*. 107–114 (1965).

17 Sonneborn, D Thrombocytopenia and iron deficiency Ann. intern. Med. *80* 111 (1974).

18 Weinfeld, A., Branehog, I., and Kutti, J Platelets in the myeloproliferative syndrome. Clin. Haematol. *4* 373–392 (1975).

Received. March 27 1979
Accepted. May 10, 1979

Dr D Shaw Department of Pharmacology and Therapeutics, University of Dundee, Ninewells Hospital, Dundee DD2 1UB (Scotland)

Acta haemat. 62, 229–235 (1979)

Anti Lymphocyte Antibody Levels in Chronic Lymphocytic Leukaemia

C. M. Lewis and G. D. Pegrum

Haematology Department, Charing Cross Hospital Medical School, London

Key Words.

Abstract. A radioimmunoassay for measuring levels of lymphocyte autoantibody in chronic lymphocytic leukaemia (CLL) has been developed. Antibody in the form of crude IgG was extracted from patients' sera and iodinated. The assay utilizes its cross-reactivity with other CLL cells. Levels were measured in 23 patients. The results show that an inverse relationship exists between the quantity of circulating CLL autoantibodies and the number of mouse red blood cell rosetting lymphocytes (M cells). The preliminary findings do not correlate with disease activity although it is our impression that patients who are maintaining higher levels of autoantibody and fewer M rosetting cells have non-progressive disease.

Introduction

Although the humoral immune response is considered to be impaired in chronic lymphocytic leukaemia (CLL), autoimmune antibodies with specificity for red cells and platelets are likely to occur [3, 4] and it has also been noted that cytotoxic antibodies to the leukaemic cells may develop [10]. We have found that antilymphocyte antibodies present in sera from these patients affect the behaviour of the CLL cells and that these antibodies cross-react extensively from patient to patient [9]. Their biological activity can be demonstrated *in vitro* through the enhancement of leukaemic cell viability and this suggested that lymphocyte antibodies may be responsible for longevity of CLL cells *in vivo*.

The extensive cross-reactivity of sera from these patients supports the idea of a common leukaemic antigen [11], but more important it was considered that this could be used to develop a radioimmunoassay. This would enable us to quantify the amount of antileukaemic antibodies and determine whether the level in different patients bears any relationship to their clinical condition or other parameters. This paper describes the method we developed and the results found

sponse to oral and parenteral iron. Pediatrics *34* 315–323 (1964).
9 Harker L. A.. The kinetics of platelet production and destruction in man. Clin. Haematol. *6* 671–693 (1977)
10 International Committee for Standardization in Haematology Br J Haemat. *25* 801–814 (1973)
11 Karpatkin, S., Garg, S. K., and Freedman, M. L.. Role of iron as a regulator of thrombopoiesis. Am. J Med. *57* 521–525 (1974)
12 Knizley H. and Noyes, W D.. Iron deficiency anaemia, papilloedema, thrombocytosis and transient hemiparesis. Archs intern. Med. *129* 483–486 (1972).
13 Penington, D G Streatfield, K., and Roxburgh, A. E.. Megakaryocytes and the heterogeneity of circulating platelets. Br J Haemat. *34* 639–652 (1976)
14 Sahud, M. A., Olsen, H., and Pedemont, L. Thrombocytopenia and iron deficiency Ann. intern. Med. *81* 132–133 (1974)
15 Scher H. and Silber R.. Iron responsive thrombocytopenia. Ann. intern. Med. *8*[illegible] 571–572 (1976)
16 Schloesser L. L., Kipp, M A., and Wenzel, F J Thrombocytosis in iron deficiency anaemia. J Lab. clin. Med. *66*. 107–114 (1965).
17 Sonneborn, D Thrombocytopenia and iron deficiency Ann. intern. Med. *80* 111 (1974).
18 Weinfeld, A., Branehog, I., and Kutti, J Platelets in the myeloproliferative syndrome. Clin. Haematol. *4* 373–392 (1975).

Received. March 27 1979
Accepted. May 10, 1979

Dr D Shaw Department of
Pharmacology and Therapeutics,
University of Dundee, Ninewells Hospital,
Dundee DD2 1UB (Scotland)

Table I. Comparison of lymphocyte subpopulations and autoantibodies in 23 patients with CLL

Patient	Total M $10^9/l$	Total N $10^9/l$	Total T $10^9/l$	Total B $10^9/l$	WBC $10^9/l$	L	Total L $10^9/l$	Auto-antibody
1	1.4	9.0	0.3	0.5	16.5	70	11.5	0.462
2	53.0	52.1	2.9	2.7	115.0	97	111.0	0.165
3	2.4	4.3	1.2	1.6	19.3	50	9.6	0.443
4	12.5	26.0	2.4	2.6	46.6	94	43.8	0.322
5	[illegible]	28.3	9.7	7.5	49.8	96	47.8	0.784
6	2.6	5.6	2.5	0.9	14.6	82	11.9	0.540
7	1.0	0.9	1.2	0.8	8.0	51	4.1	0.257
8	7.9	6.1	2.9	3.1	25.3	80	20.2	0.173
9	3.5	11.8	13.4	9.1	40.0	95	38.0	0.255
10	16.1	284.0	16.6	6.5	344.0	94	323.3	0.081
11	3.1	5.0	0.6	0.7	11.5	85	9.7	0.242
12	2.0	2.0	0.9	0.7	10.6	55	5.8	0.373
13	1.9	7.8	39.2	20.6	78.5	89	69.8	0.315
14	3.4	13.1	0.9	1.3	27.1	70	18.9	0.[illegible]40
15	58.3	45.3	1.9	1.3	129.0	83	107.0	0.215
16	2.9	10.2	0.6	0.4	17.5	82	14.3	0.370
17	3.9	6.5	1.0	1.3	16.8	72	12.0	0.352
18	4.2	12.2	1.5	0.8	24.0	78	18.7	0.220
19	1.6	2.0	1.8	0.9	9.0	73	6.5	0.415
20	4.8	6.9	1.0	1.0	17.7	79	13.9	0.320
21	12.7	22.8	0.3	0.3	40.4	90	36.3	0.201
22	4.9	59.3	2.4	3.5	71.0	99	70.2	0.367
23	1.7	2.8	0.7	1.2	8.5	78	6.6	0.432

The result in each case is the average of 3 taken over 2- to 3-month period.

Table II. Correlation coefficients between various individual parameters in the CLL patients

	M	Null	T	B	Total lymphocytes	Auto-antibodies
M	1					
Null	0.7083	1				
T	0.0420	0.4021	1			
B	0.1290	0.3900	0.9342	1		
Total lymphocytes	0.7333	0.9006	0.6120	0.6109	1	
Autoantibodies	−0.5594	−0.2158	−0.0213	0.0099	−0.3428	1

Interesting features emerged regarding the various lymphocyte populations and the levels of lymphocyte autoantibodies. Thus, there is close correlation between the numbers of T and B (E and EAC) cells ($r = 0.9342$) and between the total lymphocyte count and the numbers of lymphocytes without markers ('null') ($r = 0.9006$) and

in a group of 23 patients. In addition the variation in levels during the course of the disease was followed in 4 individuals.

Materials and Methods

Lymphocyte Preparations

Both leukaemic and healthy lymphocytes were separated from peripheral blood by centrifugation on Lymphoprep (Nyegaard, Oslo). They were washed thoroughly four times with phosphate buffered saline prior to use. For the radioimmunoassay cells were suspended in phosphate-buffered saline containing 0.1% sodium azide.

All serum samples were obtained from clotted samples of venous blood and were stored at –20 °C.

Characterization of Patients' Lymphocytes

E, EAC and M rosettes were determined by standard rosetting techniques described previously [12]. The relative proportion of lymphocytes was calculated from routine haematological investigations on a patient's blood when attending hospital clinics.

Preparation of ^{125}I γ-Globulin

Antibody was extracted from patients' sera using the A 50 Sephadex method described by *Webb* [13]. The extract was concentrated by vacuum dialysis to a volume equivalent to the volume of serum used.

A pool of this γ-globulin consisting of equal proportions of preparations from 4 different patients was used for iodination. A modification of the chloramine T method of *Greenwood et al.* [5] and *Hunter and Greenwood* [6] using 1 mCi/140 mg protein was used to label the γ-globulin with ^{125}I [7].

Radioimmunoassay of CLL Antibodies

This was performed using a method which involved the inhibition of binding of ^{125}I-labelled antibodies extracted from the CLL sera.

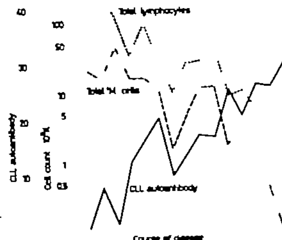
Assays were performed in a microtitre plate so that each determination was made in duplicate. Each well contained 20 μl of the appropriate dilution of patient's serum (neat, 1/4 and 1/8 dilutions were used) together with 0.1 ml of a cell suspension at a concentration of 5×10^7/ml. The cell suspension consisted of equal proportions of cells from at least 3 different patients. It was hoped that pooling cells in this way would help to enhance the common antigens of these cells in a manner similar to that suggested by *Bach et al.* [1]. Cells and serum dilution were then incubated for a period of 1 h at 37 °C. After this primary incubation, 10 μl of an appropriate dilution of ^{125}I-labelled antibodies was added and the plates incubated for a further period of 1 h at 37 °C. The plates were then harvested using a Skatron semi-automatic sample harvester and the counts from each well measured using the appropriate channel on a gamma counter.

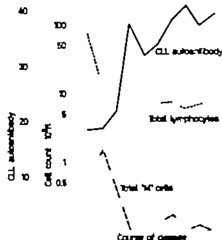
After subtraction of a blank value (cells + mixed human serum + ^{125}I-antibody) the values for the neat, 1/4 and 1/8 dilutions were plotted and the slope was taken as a measure of CLL factor. Units are therefore arbitrary.

In serial studies all serum samples from 1 patient were assayed at the same time using exactly the same mixture of cells and ^{125}I-antibody dilution. When comparing serum samples from several patients, the same method was adopted to standardize the results as much as possible. Certain CLL sera were used repeatedly and were considered to be 'standard'. Values obtained using these, enabled one experiment to be compared with another.

Results

Values of total counts, relative proportions of different types of lymphocytes and arbitrary units relating to quantities of CLL autoantibodies in 23 patients are compared in table I. These results are considered to be typical for each patient and are an average of three sequential values taken over a period of 2–3 months. They represent a period in the patient's illness when the condition was clinically stable and the haematological parameters were fairly constant. The findings were then compared statistically and the results are shown in table II.





antibody levels for that particular patient. However, a single determination of the precise levels of M cells or the antibody level as in table I does not provide evidence as to the state of the disease. 2 of the patients with the highest number of M cells and low autoantibody remain in a reasonably steady stage of their disease.

Discussion

We have developed a radioimmunoassay to measure accurately the autoantibody to leukaemic lymphocytes. This depends upon the cross-reactivity of the antibody between individual patients with chronic lymphocytic leukaemia. Although it is likely that the avidity may be variable, the technique allowed us to compare levels in several patients using the same compote of cells and a single preparation of labelled immunoglobulin. In addition we were able to measure the changes during the course of the disease using sera which had been collected from patients over several years.

We thought that the levels of lymphocyte autoantibody might be related to white count if, as we anticipated, this had an enhancing function. The inverse correlation was most unexpected although this only became really significant when related to the 'M'-rosetting lymphocytes ($r = -0.56$, $p = 0.0015$). This finding does not necessarily rule out an enhancing function for the antibody and it may suggest that antibody is being taken up from the serum by the lymphocytes, most probably by the M cells. Evidence from 'washing experiments' shows that antibody can be removed from CLL cells and this both reduces their viability and the 'M'-rosetting capacity [9].

The same procedure does not affect the numbers of T and B (E and EAC) lymphocytes [*Thompson*, personal observation]. M cells would, therefore, appear to develop their distinctive marker by the adherence of autoantibody which has affinity for the leukaemic lymphocytes, its attachment to mouse red blood cells being coincidental. Most sera from patients with this condition contains a mouse red blood cell agglutino-

also with the M cells (r = 0 7533) despite the marked variation of the total white blood cell count between individuals. The level of CLL autoantibodies does not seem to be re lated in any way to numbers of T or B (E and EAC) lymphocytes, but concentrations of autoantibodies appear to have an inverse relationship with the total numbers of M cells (r = –0.56) the total lymphocyte count (r = –0 34) and less strongly with the numbers of 'null lymphocytes (r = –0.22). When the proportion of circulating M cells is high, the amount of CLL autoantibodies is low and conversely when the number of M cells in the peripheral circulation is low the value obtained for autoantibodies is relatively high.

We studied the sera taken from patients over several years and found that when the total count and subpopulations of lymphocytes remained fairly constant there was little change in the level of anti-CLL antibodies. 4 patients who showed considerable fluctuation in numbers of white cells were also followed over a similar period. During this time each required therapy and could, therefore, be regarded as having more progressive disease. The results are given in figures 1–4 In each of the patients illustrated it was apparent that when antibody levels were high and the 'M' rosettes low the patient was usually in a satisfactory state. Active disease requiring treatment was accompanied by a high 'M' rosette count and low auto-

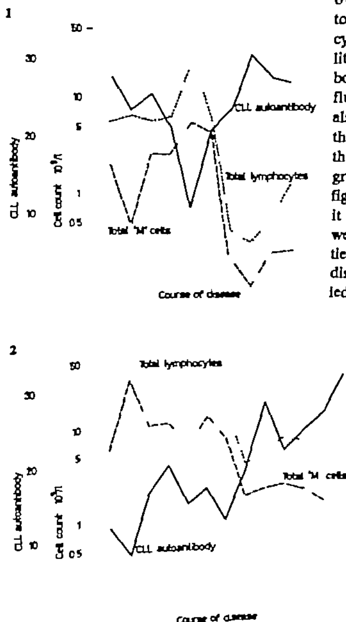


Fig. 1–4 Changes in total lymphocyte count (. .); CLL autoantibodies (—), and numbers of peripheral 'M' cells (–) during the course of the disease in 4 patients with CLL.

lymphocytes T dans la leucemie lymphocytaire chronique. Nouv Presse méd. 4 2599–2601 (1975).
9 Lewis, C. M. and Pegrum, G. D., Autoimmune antibodies in chronic lymphatic leukaemia. Br J. Haemat. 38 75–84 (1978).
10 Metzgar R. S.; Mohanakumar T and Miller D. S. Membrane-bound immunoglobulins on human leukaemic cells. Evidence for humoral immune responses of patients to leukaemia-associated antigens. J clin. Invest. 56. 331 339 (1975).
11 Pegrum, G D., Balfour, I. C., Evans, C. A., and Middleton, V L. HLA typing of 'leukaemic' cells. Lancet i. 852 (1971).
12 Pegrum, G. D. and Evans, C. A. The significance of mouse red cell rosette-forming lymphocytes in chronic lymphatic leukaemia. Acta haemat. 60 36–45 (1978).
13 Webb, A. J.: A 30-minute preparative method for isolation of IgG from human serum. Vox Sang. 23 279–286 (1972).

Received: December 27 1978
Accepted. April 20, 1979

C. M. Lewis, Haematology Department, Charing Cross Hospital Medical School, Fulham Palace Road, London, W6 8RF (England)

gen, and although titration is somewhat less satisfactory than the radioimmunoassay the values obtained show some agreement with autoantibody levels measured here [personal observations with *C A Evans*]

CLL lymphocyte antibody is present in sera from all the patients studied so far and we now need to know whether this has any relevance to the disease state. Our initial observations suggest that the comparative level in 23 patients is not directly related to the clinical manifestations, but no patients with rapidly progressive disease were included. In the serial studies we were able to identify episodes when the inverse relationship between the autoantibody and 'M' rosetting cells was greatest. At these times the disease appeared to be most quiescent and required no treatment. Further work is necessary to determine more precisely the possible clinical significance of the lymphocyte antibody level

Our results do not provide any information regarding the origin of the CLL lymphocyte antibody whether it is part of the disease process or an immune response to a malignancy It may be relevant to reiterate the close association between numbers of T and B lymphocytes and their relationship to the total white count. It has already been suggested that elevated numbers of T cells are a response to the disease [2, 8] and elevated numbers of antibody-producing cells have also been described [*Vanhegan* personal commun.] The values we have obtained suggest that both normal lymphocyte compartments may be equally elevated in response to the leukaemia.

The majority of peripheral lymphocytes in CLL show 'M' or null characteristics with respect to rosetting techniques yet the majority are also monoclonal with respect to surface immunoglobulin. Further examination of the properties of the lymphocyte autoantibody may help this apparent contradiction.

Some leukaemic cells probably pass into the blood from extracirculatory lymphoid tissues and the presence of antibody may augment or inhibit this process. If this is so, the levels of the autoantibody might be a more useful parameter to relate to changes in clinical condition than the individual populations of lymphocytes. Certainly the CLL lymphocyte antibody levels we have measured so far show less variation in the steady-state than the level of 'M'-rosetting cells.

References

1 Bach, M., Bach, F H., and Zarling, J Pool-priming – a means of generating T lymphocytes cytotoxic to tumour viruses. Lancet I: 20–21 (1978)

2 Catovsky D Milliani, E., Okos, A., and Galton, D A. G Clinical significance of T cells in chronic lymphocytic leukaemia. Lancet II: 751–753 (1974)

3 Dacie, J V The haemolytic anaemias, part III 2nd ed., p 730 (Churchill, London 1967).

4 Ebbe, S., Wittels, B., and Dameshek, W Autoimmune thrombocytopaenic purpura (ITP type) with chronic lymphatic leukaemia. Blood *19* 23–37 (1962).

5 Greenwood, F C., Hunter W M., and Glover J S., The preparation of ¹³¹I-labelled human growth hormone of high specific activity Biochem. J *89* 114–123 (1963).

6 Hunter W H. and Greenwood, F C. Preparation of iodine 131 labelled human growth hormone of high specific activity Nature, Lond. *194* 495–496 (1962)

7 Hutchinson, H. D and Zeigler D. W.. Criteria for preparing evaluating and standardising iodinated globulins for radioimmunoassay procedures. Appl. Microbiol. 228: 935–942 (1974).

8 Lang, J M., Oberling, F., Schille, A. C. et Mayer S Elévation du chiffre absolu des

common. The diagnosis of β-thalassaemia trait was based on the existence of chronic microcytic hypochromic anaemia with a haemoglobin A_2 concentration of more than 3.2% on cellulose-acetate electrophoresis. The diagnosis of iron deficiency anaemia was established by an initial transferrin saturation of less than 15%. At the time of the study some of these patients were already on oral iron therapy and had normal or increased serum iron levels. The normal controls were healthy laboratory and hospital personnel aged 20–60 years. Blood samples were collected in the non-fasting state.

Blood counts were performed on a Coulter model S counter standardized with the 4C Coulter cell control. Serum ferritin levels were assayed using the immunoradiometric assay of *Miles et al.* [6]. Serum iron was determined by colorimetric analysis as recommended by the ICSH [5] and the serum-unsaturated iron-binding capacity was measured by the magnesium carbonate absorption method [2]. The total iron-binding capacity (TIBC) was derived from the sum of serum iron and unsaturated iron-binding capacity. I comparing the frequency of abnormal laboratory values in the different groups of patients, haemoglobin level below 12 g/dl, MCV below 80 μm^3 serum ferritin of less than 16 ng/ml, serum iron less than 60 ng/dl, transferrin saturation below 15%, and TIBC over 450 ng/dl were considered as abnormal. These were arbitrary values chosen for the sake of comparison between the different groups and are not intended to represent the established limits of the normal range.

Results

The mean haemoglobin, MCV serum ferritin, serum iron, TIBC, and percent transferrin saturation in normal males, females, and patients with β-thalassaemia minor and iron deficiency are presented in table I. Haemoglobin, MCV and serum ferritin were significantly lower in normal females than in normal males ($p < 0.001$). On the other hand, no significant sex difference could be shown in mean serum iron, TIBC, and transferrin saturation in the normal control population. When compared with the normal controls, the thalassaemic and iron-deficient groups had a significant reduction in their MCV and mean haemoglobin levels ($p < 0.001$) In the thalassaemic group, the mean serum ferritin, serum iron, TIBC, and transferrin saturation were identical with the corresponding mean values of the normal control groups. In contrast, in the iron-deficient group, serum ferritin, serum iron, and transferrin saturation were lower and the TIBC higher than in the normal control or thalassaemic groups ($p < 0.001$).

In order to estimate the ability of the different laboratory parameters listed in table I to distinguish between individual subjects within the normal, thalassaemic and iron-

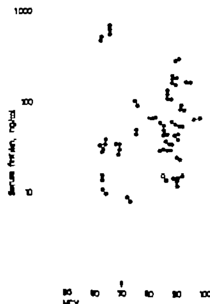


Fig. 1. Correlation between serum ferritin and MCV ● = normal, O = iron deficiency and O β-thalassaemia trait.

Acta haemat. 62 236–239 (1979)

Serum Ferritin and Mean Corpuscular Volume Measurement in the Diagnosis of β-Thalassaemia minor and Iron Deficiency

Chaim Hershko Abraham M Konijn and Alvar Loria

Department of Haematology Hadassah University Hospital, and Department of Nutrition, Hebrew University Hadassah Medical School, Jerusalem, and Instituto Nacional de la Nutrición, Mexico City DF

Key Words. Iron deficiency Serum ferritin β-Thalassaemia minor

Abstract. The value of serum ferritin and mean corpuscular volume (MCV) measurement in distinguishing between iron deficient, β-thalassaemia trait and normal subjects has been studied. Normal subjects had normal ferritin and MCV iron-deficient ones had low ferritin and low or normal MCV and thalassaemics had normal ferritin and low MCV By the combined use of these two measurements it was possible to identify individuals belonging to one of the three categories with an accuracy of over 95%. Although definitive diagnosis of β thalassaemia trait requires the demonstration of abnormal haemoglobin A_2 levels or reduced β-chain synthesis, serum ferritin and MCV measurements are useful screening procedures for the initial diagnosis of β-thalassaemia trait and iron deficiency Because of the very small amounts of blood required for both of these measurements, they are particularly suitable for surveying large numbers of subjects in populations with a high prevalence of hypochromic microcytic anaemias.

Both iron deficiency and β-thalassaemia trait are common causes of anaemia in people of Mediterranean and Middle Eastern origin. Microcytosis is a common feature of both types of anaemia and further distinction between the two conditions is usually made by haemoglobin electrophoresis, serum iron, and transferrin saturation measurements. In the present report we wish to describe the simultaneous use of the mean corpuscular volume (MCV) and serum ferritin measurement in distinguishing iron deficiency from β-thalassaemia trait among patients with microcytic anaemia.

Patients and Methods

50 patients with β-thalassaemia trait (36 females and 14 males) were studied together with 39 patients with iron deficiency (33 females and 6 males) and 87 normal subjects (54 females and 33 males). All patients with β-thalassaemia trait were from families who emigrated to Israel from Iraq and Iran among whom β-thalassaemia is very

common. The diagnosis of β-thalassaemia trait was based on the existence of chronic microcytic hypochromic anaemia with a haemoglobin A_2 concentration of more than 3.2% on cellulose-acetate electrophoresis. The diagnosis of iron deficiency anaemia was established by an initial transferrin saturation of less than 15% At the time of the study some of these patients were already on oral iron therapy and had normal or increased serum iron levels. The normal controls were healthy laboratory and hospital personnel aged 20–60 years. Blood samples were collected in the non-fasting state.

Blood counts were performed on a Coulter model S counter standardized with the 4C Coulter cell control. Serum ferritin levels were assayed using the immunoradiometric assay of *Miles et al.* [6]. Serum iron was determined by colorimetric analysis as recommended by the ICSH [5] and the serum-unsaturated iron-binding capacity was measured by the magnesium carbonate absorption method [2]. The total iron-binding capacity (TIBC) was derived from the sum of serum iron and unsaturated iron-binding capacity. In comparing the frequency of abnormal laboratory values in the different groups of patients, haemoglobin level below 12 g/dl, MCV below 80 μm serum ferritin of less than 16 ng/ml, serum iron less than 60 µg/dl, transferrin saturation below 15%, and TIBC over 450 µg/dl were considered as abnormal. These were arbitrary values chosen for the sake of comparison between the different groups and are not intended to represent the established limits of the normal range.

Results

The mean haemoglobin, MCV serum ferritin, serum iron, TIBC, and percent transferrin saturation in normal males, females, and patients with β-thalassaemia minor and iron deficiency are presented in table I. Haemoglobin, MCV and serum ferritin were significantly lower in normal females than in normal males ($p < 0.001$). On the other hand, no significant sex difference could be shown in mean serum iron, TIBC, and transferrin saturation in the normal control population. When compared with the normal controls, the thalassaemic and iron-deficient groups had a significant reduction in their MCV and mean haemoglobin levels ($p < 0.001$). In the thalassaemic group, the mean serum ferritin, serum iron, TIBC, and transferrin saturation were identical with the corresponding mean values of the normal control groups. In contrast, in the iron-deficient group, serum ferritin, serum iron, and transferrin saturation were lower and the TIBC higher than in the normal control or thalassaemic groups ($p < 0.001$).

In order to estimate the ability of the different laboratory parameters listed in table I to distinguish between individual subjects within the normal, thalassaemic and iron-

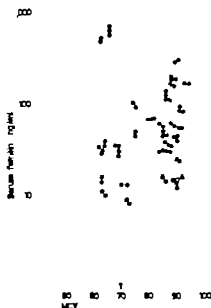


Fig. 1. Correlation between serum ferritin and MCV ● = normal; O = iron deficiency and ○ β-thalassaemia trait.

Acta haemat. 62: 236–239 (1979)

Serum Ferritin and Mean Corpuscular Volume Measurement in the Diagnosis of β-Thalassaemia minor and Iron Deficiency

Chaim Hershko, Abraham M. Konijn and Alvar Loria

Department of Haematology, Hadassah University Hospital, and Department of Nutrition, Hebrew University Hadassah Medical School, Jerusalem, and Instituto Nacional de la Nutrición, Mexico City, DF

Key Words. Iron deficiency · Serum ferritin · β-Thalassaemia minor

Abstract. The value of serum ferritin and mean corpuscular volume (MCV) measurement in distinguishing between iron deficient, β thalassaemia trait and normal subjects has been studied. Normal subjects had normal ferritin and MCV. Iron-deficient ones had low ferritin and low or normal MCV, and thalassaemics had normal ferritin and low MCV. By the combined use of these two measurements it was possible to identify individuals belonging to one of the three categories with an accuracy of over 95%. Although definitive diagnosis of β-thalassaemia trait requires the demonstration of abnormal haemoglobin A_2 levels or reduced β-chain synthesis, serum ferritin and MCV measurements are useful screening procedures for the initial diagnosis of β-thalassaemia trait and iron deficiency. Because of the very small amounts of blood required for both of these measurements they are particularly suitable for surveying large numbers of subjects in populations with a high prevalence of hypochromic microcytic anaemias.

Both iron deficiency and β-thalassaemia *trait* are common causes of anaemia in people of Mediterranean and Middle Eastern origin. Microcytosis is a common feature of both types of anaemia and further distinction between the two conditions is usually made by haemoglobin electrophoresis, serum iron, and transferrin saturation measurements. In the present report we wish to describe the simultaneous use of the mean corpuscular volume (MCV) and serum ferritin measurement in distinguishing iron deficiency from β-thalassaemia trait [illegible] patients with microcytic anaemia.

Patients and Methods

50 patients with β-thalassaemia trait [illegible] males and 14 males) were studied together with [illegible] patients with iron deficiency (33 females [illegible] males) and 87 normal subjects (54 females and [illegible] males). All patients with β-thalassaemia trait [illegible] from families who emigrated to Israel from [illegible] and Iran among whom β-thalassaemia is [illegible]

common. The diagnosis of β-thalassaemia trait was based on the existence of chronic microcytic hypochromic anaemia with haemoglobin A_2 concentration of more than 3.2% on cellulose-acetate electrophoresis. The diagnosis of iron deficiency anaemia was established by an initial transferrin saturation of less than 15%. At the time of the study some of these patients were already on oral iron therapy and had normal or increased serum iron levels. The normal controls were healthy laboratory and hospital personnel aged 20–60 years. Blood samples were collected in the non-fasting state.

Blood counts were performed on Coulter model S counter standardized with the 4C Coulter cell control. Serum ferritin levels were assayed using the immunoradiometric assay of *Miles et al.* [6]. Serum iron was determined by colorimetric analysis as recommended by the ICSH [5] and the serum-unsaturated iron-binding capacity was measured by the magnesium carbonate absorption method [2]. The total iron-binding capacity (TIBC) was derived from the sum of serum iron and unsaturated iron-binding capacity. In comparing the frequency of abnormal laboratory values in the different groups of patients, haemoglobin level below 12 g/dl, MCV below 80 μm^3 serum ferritin of less than 16 ng/ml, serum iron less than 60 ng/dl, transferrin saturation below 15%, and TIBC over 450 ng/dl were considered as abnormal. These were arbitrary values chosen for the sake of comparison between the different groups and are not intended to represent the established limits of the normal range.

Results

The mean haemoglobin, MCV serum ferritin, serum iron, TIBC, and percent transferrin saturation in normal males, females, and patients with β-thalassaemia minor and iron deficiency are presented in table I. Haemoglobin, MCV and serum ferritin were significantly lower in normal females than in normal males ($p < 0.001$). On the other hand, no significant sex difference could be shown in mean serum iron, TIBC, and transferrin saturation in the normal control population. When compared with the normal controls, the thalassaemic and iron-deficient groups had a significant reduction in their MCV and mean haemoglobin levels ($p < 0.001$). In the thalassaemic group, the mean serum ferritin, serum iron, TIBC, and transferrin saturation were identical with the corresponding mean values of the normal control groups. In contrast, in the iron-deficient group, serum ferritin, serum iron, and transferrin saturation were lower and the TIBC higher than in the normal control or thalassaemic groups ($p < 0.001$)

In order to estimate the ability of the different laboratory parameters listed in table I to distinguish between individual subjects within the normal, thalassaemic and iron

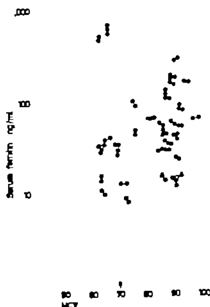


Fig. 1. Correlation between serum ferritin and MCV ● = normal, ○ = iron deficiency and ○ β-thalassaemia trait.

Acta haemat. 62: 236–239 (1979)

Serum Ferritin and Mean Corpuscular Volume Measurement in the Diagnosis of β-Thalassaemia minor and Iron Deficiency

Chaim Hershko, Abraham M. Konijn and Alvar Loria

Department of Haematology, Hadassah University Hospital, and Department of Nutrition, Hebrew University Hadassah Medical School, Jerusalem, and Instituto Nacional de la Nutrición, Mexico City, DF

Key Words. Iron deficiency · Serum ferritin · β-Thalassaemia minor

Abstract. The value of serum ferritin and mean corpuscular volume (MCV) measurement in distinguishing between iron deficient, β thalassaemia trait and normal subjects has been studied. Normal subjects had normal ferritin and MCV, iron-deficient ones had low ferritin and low or normal MCV, and thalassaemics had normal ferritin and low MCV. By the combined use of these two measurements it was possible to identify individuals belonging to one of the three categories with an accuracy of over 95%. Although definitive diagnosis of β-thalassaemia trait requires the demonstration of abnormal haemoglobin A_2 levels or reduced β-chain synthesis, serum ferritin and MCV measurements are useful screening procedures for the initial diagnosis of β-thalassaemia trait and iron deficiency. Because of the very small amounts of blood required for both of these measurements, they are particularly suitable for surveying large numbers of subjects in populations with a high prevalence of hypochromic microcytic anaemias.

Both iron deficiency and β-thalassaemia trait are common causes of anaemia in people of Mediterranean and Middle Eastern origin. Microcytosis is a common feature of both types of anaemia and further distinction between the two conditions is usually made by haemoglobin electrophoresis, serum iron, and transferrin saturation measurements. In the present report we wish to describe the simultaneous use of the mean corpuscular volume (MCV) and serum ferritin measurement in distinguishing iron deficiency from β-thalassaemia trait among patients with microcytic anaemia.

Patients and Methods

50 patients with β-thalassaemia trait (36 females and 14 males) were studied together with 39 patients with iron deficiency (33 females and 6 males) and 87 normal subjects (54 females and 33 males). All patients with β-thalassaemia trait were from families who emigrated to Israel from Iraq and Iran among whom β-thalassaemia is very

common. The diagnosis of β-thalassaemia trait was based on the existence of chronic microcytic hypochromic anaemia with haemoglobin A_2 concentration of more than 3.2% on cellulose-acetate electrophoresis. The diagnosis of iron deficiency anaemia was established by an initial transferrin saturation of less than 15%. At the time of the study some of these patients were already on oral iron therapy and had normal or increased serum iron levels. The normal controls were healthy laboratory and hospital personnel aged 20–60 years. Blood samples were collected in the non-fasting state.

Blood counts were performed on Coulter model S counter standardized with the 4C Coulter cell control. Serum ferritin levels were assayed using the immunoradiometric assay of *Miles et al.* [6]. Serum iron was determined by colorimetric analysis as recommended by the ICSH [5] and the serum-unsaturated iron-binding capacity was measured by the magnesium carbonate absorption method [7]. The total iron-binding capacity (TIBC) was derived from the sum of serum iron and unsaturated iron-binding capacity. In comparing the frequency of abnormal laboratory values in the different groups of patients, haemoglobin level below 12 g/dl, MCV below 80 μm^3, serum ferritin of less than 16 ng/ml, serum iron less than 60 ng/dl, transferrin saturation below 15%, and TIBC over 450 μg/dl were considered as abnormal. These were arbitrary values chosen for the sake of comparison between the different groups and are not intended to represent the established limits of the normal range.

Results

The mean haemoglobin, MCV serum ferritin, serum iron, TIBC, and percent transferrin saturation in normal males, females, and patients with β-thalassaemia minor and iron deficiency are presented in table I. Haemoglobin, MCV and serum ferritin were significantly lower in normal females than in normal males ($p < 0.001$). On the other hand, no significant sex difference could be shown in mean serum iron, TIBC, and transferrin saturation in the normal control population. When compared with the normal controls, the thalassaemic and iron-deficient groups had a significant reduction in their MCV and mean haemoglobin levels ($p < 0.001$). In the thalassaemic group, the mean serum ferritin, serum iron, TIBC, and transferrin saturation were identical with the corresponding mean values of the normal control groups. In contrast, in the iron-deficient group, serum ferritin, serum iron, and transferrin saturation were lower and the TIBC higher than in the normal control or thalassaemic groups ($p < 0.001$).

In order to estimate the ability of the different laboratory parameters listed in table I to distinguish between individual subjects within the normal, thalassaemic and iron-

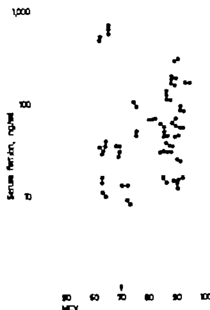


Fig. 1. Correlation between serum ferritin and MCV ● = normal; O = iron deficiency and O β-thalassaemia trait.

Acta haemat. 62, 236–239 (1979)

Serum Ferritin and Mean Corpuscular Volume Measurement in the Diagnosis of β-Thalassaemia minor and Iron Deficiency

Chaim Hershko, Abraham M. Konijn and Alvar Loria

Department of Haematology, Hadassah University Hospital, and Department of Nutrition, Hebrew University Hadassah Medical School, Jerusalem, and Instituto Nacional de la Nutrición, Mexico City, DF

Key Words. Iron deficiency · Serum ferritin · β-Thalassaemia minor

Abstract. The value of serum ferritin and mean corpuscular volume (MCV) measurement in distinguishing between iron deficient, β-thalassaemia trait and normal subjects has been studied. Normal subjects had normal ferritin and MCV; iron-deficient ones had low ferritin and low or normal MCV, and thalassaemics had normal ferritin and low MCV. By the combined use of these two measurements it was possible to identify individuals belonging to one of the three categories with an accuracy of over 95%. Although definitive diagnosis of β-thalassaemia trait requires the demonstration of abnormal haemoglobin A_2 levels or reduced β-chain synthesis, serum ferritin and MCV measurements are useful screening procedures for the initial diagnosis of β-thalassaemia trait and iron deficiency. Because of the very small amounts of blood required for both of these measurements, they are particularly suitable for surveying large numbers of subjects in populations with a high prevalence of hypochromic microcytic anaemias.

Both iron deficiency and β-thalassaemia trait are common causes of anaemia in people of Mediterranean and Middle Eastern origin. Microcytosis is a common feature of both types of anaemia and further distinction between the two conditions is usually made by haemoglobin electrophoresis, serum iron, and transferrin saturation measurements. In the present report we wish to describe the simultaneous use of the mean corpuscular volume (MCV) and serum ferritin measurement in distinguishing iron deficiency from β-thalassaemia trait among patients with microcytic anaemia.

Patients and Methods

50 patients with β-thalassaemia trait (36 females and 14 males) were studied together with 39 patients with iron deficiency (33 females and 6 males) and 87 normal subjects (54 females and 33 males). All patients with β-thalassaemia trait were from families who emigrated to Israel from Iraq and Iran among whom β-thalassaemia is very

TIBC		Saturation	
μg/dl	% > 450	%	% < 15
364 ± 56	6.0	32.1 ±12.5	3.1
376 ± 68	13.0	30.1 ± 8.1	3.8
363 ± 82	20.0	30.3 ±13.9	14.0
432 ± 73	48.7	18.1 ±13.4	59.0

Low serum iron and transferrin saturation and increased TIBC are characteristic of iron deficiency and are commonly used for its diagnosis [1]. However, serum iron may be affected by external contamination of blood samples, by diurnal variation, and by concurrent iron therapy, whereas serum ferritin is independent of these variables. Since serum ferritin is invariably low in iron deficiency, its measurement allows an easy distinction between iron deficiency anaemia and other forms of microcytic anaemia such as thalassaemia.

In line with an earlier report [4] we found that the majority of patients with thalassaemia trait had normal serum ferritin levels. However, in 8 of our 50 thalassaemic patients serum ferritin was 500 ng/ml or higher and in 7 thalassaemic patients with a transferrin saturation of less than 15%, the mean serum ferritin was 29 ng/ml, ranging from 14 to 50 ng/ml. Thus, a slight increase in iron stores may be associated with β-thalassaemia trait and is probably explained by the enhancement of iron absorption found in some haemolytic anaemia [3] and a shift of red blood cell iron into reticuloendothelial organs.

References

1 Bothwell, T. H. and Finch, C. A.: Iron metabolism (Little, Brown, Boston 1962).
2 Cook, J. D.: An evaluation of absorption methods for measurement of plasma iron binding capacity. J. Lab. clin. Med. 76: 497–506 (1970).
3 Hershko, C.: Storage iron regulation; in Brown, Progress in hematology, vol. X, pp. 105–148 (Grune & Stratton, New York 1977).
4 Hussein, S., Hoffbrand, A. V., Laulicht, M., Attock, B., and Letsky, E.: Serum ferritin levels in beta-thalassemia trait. Br. med. J. ii: 920–921 (1976).
5 International Committee for Standardization in Hematology: Expert panel on iron. Proposed recommendations for measurement of serum iron in human blood. Br. J. Haemat. 20: 451–453 (1971).
6 Miles, L. C. M., Lipschitz, D. A., Bieber, C. P. and Cook, J. D.: Measurement of serum ferritin by a two-site immunoradiometric assay. Analyt. Biochem. 61: 209–224 (1974).

Received: December 21, 1978
Accepted: April 20, 1979

Prof. Chaim Hershko, Department of Haematology, Hadassah University Hospital, Jerusalem (Israel)

Table I. Laboratory data in normal, thalassaemic, and iron-deficient subjects (mean ± 1 SD)

Group	Number	Haemoglobin		MCV		Ferritin		Serum iron	
		g/dl	% < 12.0	μm^3	% < 80	ng/ml	% < 16	$\mu g/dl$	% < 60
Normal males	33	15.3 ± 1.2	0	89.3 ± 3.4	0	149.0 ± 92.5	0	117 ± 39	3.0
Normal females	54	13.9 ± 1.3	7.4	85.9 ± 4.7	0	75.8 ± 42.9	0	113 ± 25	1.9
Thalassaemia	50	11.3 ± 1.3	80.0	67.5 ± 6.4	96.0	113.6 ± 158.3	2.0	110 ± 45	10.0
Iron deficiency	39	10.0 ± 2.3	76.9	76.1 ± 10.0	69.2	9.9 ± 4.3	100.0	78 ± 61	51.8

deficient groups, the percentage of patients with reduced levels of haemoglobin, MCV ferritin, serum iron, and transferrin saturation and those with increased levels of TIBC has been determined for each experimental group. As expected, only a few subjects in the normal group of males and females had abnormal laboratory values. In contrast, many of the thalassaemic and iron deficient patients had low haemoglobin or reduced MCV. In general, the highest proportion of subjects with low serum iron, low transferrin saturation, and increased TIBC was found in the group of iron-deficient patients. However serum iron and transferrin measurements were of a limited value in identifying individual patients in the pathologic groups since in about half of the iron deficient patients and in most of the thalassaemic patients these measurements were normal. The most consistent difference between the normal, β-thalassaemic, and iron-deficient groups was found in relation to serum ferritin and MCV measurements (fig. 1). All iron-deficient patients had serum ferritin levels below 16 ng/ml as against none in the normal controls and only 2% in the thalassaemics. On the other hand, MCV was very useful in distinguishing between β-thalassaemia trait and normal subjects. All except 3 of the 50 thalassaemic subjects had low MCV as compared to none of the normal controls. A high proportion of the iron-deficient patients also had reduced MCV values, but these individuals could be easily distinguished from the thalassaemic subjects by the normal ferritin levels in the latter group.

Discussion

These data indicate that it is possible to distinguish between normal individuals, patients with iron deficiency and patients, with β-thalassaemia trait by the combined use of serum ferritin and MCV measurements with an accuracy of over 95%. Both measurements require only small amounts of blood and are, therefore, particularly suitable for surveying large numbers of subjects by means of capillary blood samples.

Acta haemat. 62, 241–246 (1979)

Oscillations of Marrow Culture Growth in Acute Myeloid Leukemia during Remission Induction and Remission

Anita Gustavsson, Tor Olofsson and Inge Olsson

Department of Internal Medicine, University Hospital, Lund

Key Words. Acute myeloid leukemia · *In vitro* culture · Sequential studies

Abstract. Sequential studies in acute myeloid leukemia of bone marrow cells in agar culture showed striking fluctuations of colony and cluster formation during induction of remission. These oscillations may be initiated by recruitment of resting leukemic and normal cells upon chemotherapeutic perturbation. Striking oscillations of the colony and cluster formation occurred also during remission with or without maintenance therapy but with a longer periodicity as compared to the phase of induction of remission. Oscillations of the marrow growth capacity during remission may reflect potential relapses where the outcome depends on proliferative advantages of the normal or leukemic cell clone.

Introduction

In acute myeloid leukemia (AML) profound abnormalities of the *in vitro* growth pattern of marrow cells in semisolid agar [2, 7] have been revealed [4–8, 9–13]. Frequently there is an absence of colonies with greater than 40 cells (CFU-C) but production of various amounts of clusters with 3–40 cells. Reclassification of AML according to *in vitro* growth pattern may have prognostic value [10]. This work presents sequential studies of the *in vitro* growth pattern in agar of marrow cells from individual AML patients undergoing pulse chemotherapy for induction of remission and for maintenance of remission. The results may be of value in understanding how remission is achieved and maintained.

Materials and Methods

Patient Selection and Treatment

Serial studies were performed during induction of remission in 8 patients with adult AML, diagnosed using the criteria of *Hayhoe and Cawley* [6]. For induction of remission 5-day courses of thioguanine (100 mg/m^2), cytosine arabinoside (100 mg/m^2) and prednisone (30 mg/m^2) were administered every 10–14 days with rubidomycin (40 mg/m^2) on the first day of each course (TRAP). TRAP alternating with cyclophosphamide (100 mg/m^2), cytosine arabinoside (100 mg/m^2) and prednisone (200 mg/day) for 5 days with vincristine (2 mg) on the first day (COAP) was

Book Review

A F W Morselt
Applications and Possibilities of Cytophotometry for Red Cell Hematology
Progress in Histochemistry and Cytochemistry
Vol. 11, No 3
Fischer Stuttgart 1978
VI + 42 pp 28 tab., DM 36.–
ISBN 3-437-10557-4

Die kurze Monographie fasst die Ergebnisse langjähriger technisch aufwendiger Untersuchungen knapp und klar zusammen. Bei oberflächlicher Betrachtung mag es scheinen, als ob nur ein kleiner Kreis von Spezialisten angesprochen werde. Vertieft man sich in *Morselts* Werk, wird bald einmal deutlich, dass es hier um eine Reihe von grundlegenden Fragen der Erythropoese geht, die jeden Hämatologen und wohl auch weitere Kreise interessieren dürften.

Es geht dem Autor darum, auf Möglichkeiten der mikrospektrophotometrischen Messung (= Messung des Absorptionsspektrums an einem Punkt) und zytophotometrischen Messung (= Integration der totalen über einer Zelle oder einer Zellorganelle bei bestimmter Wellenlänge gemessenen Extinktion) von Erythrozyten oder hämoglobinhaltigen Zellen des Menschen hinzuweisen. Verschiedene Fragen liessen sich durch mikrospektrophotometrische Untersuchungen an Erythrozyten beantworten. Einfluss verschiedenartiger Fixationen auf das Spektrum des Hämoglobins, Vergleich der Spektren von Erythrozyten von Erwachsenen und Neugeborenen, Ablauf der Malaria-Pigmentbildung in befallenen Erythrozyten, Abbau des Hämoglobins phagozytierter Erythrozyten zu Hämosiderin und Bilirubin in Makrophagen, Extinktionen elektronenoptisch definierter Strukturen und Vergleich der Absorptionsspektren des Hämoglobins und anderer Farbstoffe in Lösung und in Gewebe.

Zytophotometrische Bestimmungen des Hämoglobingehaltes einzelner Erythrozyten erlaubten, den Effekt einer Eisentherapie bei einer Eisenmangelanämie anhand der Hämoglobingehaltverteilung präzis zu verfolgen sowie den relativen Gehalt von HbF in einem einzelnen Erythrozyten zu bestimmen und zudem die Wirkung der Elution zu verfolgen.

Durch gleichzeitige zytophotometrische Bestimmung des Hämoglobingehaltes und der Projektionsfläche einzelner Erythrozyten in Erythrozytenpopulationen bekannten Alters konnte gezeigt werden, dass bei Erythrozyten mit vergleichbarem Hämoglobingehalt die Projektionsfläche mit zunehmendem Alter abnimmt. Durch Verwendung von Brillant Cresyl Blau und Messung bei 580 und 416 nm konnte gezeigt werden, dass bei einer Eisenmangelanämie nach Eisenzufuhr der Hämoglobingehalt der im Blut zirkulierenden Retikulozyten zwischen dem 3. und 5 Tag um etwa 40% zunimmt. Mit der gleichen Methode wurde gefunden, dass polychromatische Normoblasten rund doppelt soviel RNS, aber fast gleich viel Hämoglobin enthalten wie azidophile. Durch kombinierte zytophotometrische Bestimmungen des Hämoglobin- und DNS-Gehaltes einzelner Erythrozytenvorläufer verschiedener Reifegrade konnte schliesslich die Klassifizierung der kongenitalen dyserythropoetischen Anämie erleichtert werden.

Gesamthaft betrachtet, vermittelt die Monographie eine sehr wertvolle Übersicht über die vielfältigen Anwendungsmöglichkeiten mikrospektrophotometrischer und zyctophotometrischer Methoden in der Hämatologie.

H. P Wagner Bern

Original Papers

Acta haemat. 62: 241–246 (1979)

Oscillations of Marrow Culture Growth in Acute Myeloid Leukemia during Remission Induction and Remission

Anita Gustavsson, Tor Olofsson and Inge Olsson

Department of Internal Medicine, University Hospital, Lund

Key Words. Acute myeloid leukemia · *In vitro* culture · Sequential studies

Abstract. Sequential studies in acute myeloid leukemia of bone marrow cells in agar culture showed striking fluctuations of colony and cluster formation during induction of remission. These oscillations may be initiated by recruitment of resting leukemic and normal cells upon chemotherapeutic perturbation. Striking oscillations of the colony and cluster formation occurred also during remission with or without maintenance therapy but with a longer periodicity as compared to the phase of induction of remission. Oscillations of the marrow growth capacity during remission may reflect potential relapses where the outcome depends on proliferative advantages of the normal or leukemic cell clone.

Introduction

In acute myeloid leukemia (AML) profound abnormalities of the *in vitro* growth pattern of marrow cells in semisolid agar [2, 7] have been revealed [4, 8, 9, 13]. Frequently there is an absence of colonies with greater than 40 cells (CFU-C), but production of various amounts of clusters with 3–40 cells. Reclassification of AML according to *in vitro* growth pattern may have prognostic value [10]. This work presents sequential studies of the *in vitro* growth pattern in agar of marrow cells from individual AML patients undergoing pulse chemotherapy for induction of remission and for maintenance of remission. The results may be of value in understanding how remission is achieved and maintained.

Materials and Methods

Patient Selection and Treatment

Serial studies were performed during induction of remission in 8 patients with adult AML, diagnosed using the criteria of *Hayhoe and Cawley* [6]. For induction of remission 5-day courses of thioguanine (100 mg/m²), cytosine arabinoside (100 mg/m²) and prednisone (30 mg/m²) were administered every 10–14 days with rubidomycin (40 mg/m²) on the first day of each course (TRAP). TRAP alternating with cyclophosphamide (100 mg/m²), cytosine arabinoside (100 mg/m²) and prednisone (200 mg/day) for 5 days with vincristine (2 mg) on the first day (COAP) was

used as maintenance therapy Complete remission was defined as normal bone marrow cellularity with less than 5% blasts when the neutrophil counts had risen to 1,500/μl or greater and the platelet counts to at least 100,000/μl blood

Collection of Bone Marrow

2–3 ml of bone marrow was aspirated from sternum prior to each chemotherapy course or more frequently and collected in 3 ml of McCoy's medium, 100 IU of heparin and 75 U of Varidase (Lederle Laboratories, Pearl River N Y) Marrow particles were disrupted by repeated suction through a needle Nucleated cells were counted in a Bürker chamber for determination of marrow cellularity

Cell Separation

7 ml of marrow suspended in culture medium were layered on top of 5 ml Isopaque Ficoll [1] solution in 16-ml plastic tubes. The tubes were centrifuged with a swing out rotor for 10 min at 70 g and for 15 min at 700 g The cells collected on top of the Isopaque Ficoll (A cells) were removed, washed three times in culture medium and used for agar culture A cells from normal peri-

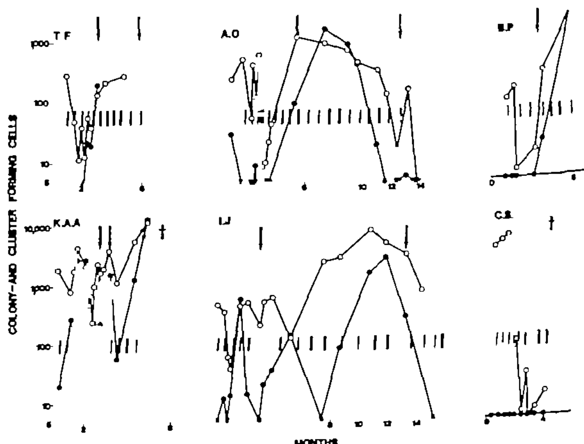


Fig. 1. Serial studies in 6 patients with AML (K.A.A., 24-year-old male; L.J 40-year-old f male; C.S., 41 year-old female; T F 26-year-old male; B P 44-year-old male; A.O 41 year-old male) of colony forming cells (●) per 10^4 and cluster forming cells (O) per 10^4 seeded marrow cells. The number of cell aggregates grown is given on a logarithmic scale The abscissa shows the time in months after diagnosis. Chemotherapy courses are shown TRAP (▯) and COAP (■) Complete remission (↓) (that of K. A. A. was however only a partial remission) and relapse (are indicated. The karyotype of marrow cells from C. S. was 45 XO (8.21) and that of T F showed Ph¹-chromosome t (9:22) the karyotype of the other patients was normal

pheral blood were used for preparation of feeder layers. Colony and cluster forming cells were quantitatively recovered (98%) in the A cell fraction.

Cell Culture Technique

The culture technique of *Pike and Robinson* [14] was utilized. The feeder layers contained 3×10^6 A cells obtained from the peripheral blood. 1 ml of cells in 0.5% agar (Difco Bacto Agar) in culture medium was plated in 35-mm Falcon plastic Petri dishes. The over layers contained 10^5 marrow A cells from the AML patients. After gelling at room temperature the plates were incubated at 37 °C in fully humidified atmosphere with 7.5% CO_2 in air.

Culture Scoring

Cultures were evaluated at 7 and 10 days for clusters of 3–40 cells and colonies of greater than 40 cells. Data presented for colony formation is from 10 days of culture while data for cluster formation is from 7 days of culture. On the basis of cell counts and differentials of the marrow aspirate and the A cell fraction used for plating, colony and cluster formation per microliter bone marrow were calculated (fig. 2). This was done as described previously [12].

Results

Figure 1 shows that a short phase of low or minimal growth of the marrow cells in agar is usually seen prior to complete remission (also seen in figures 2–3). However 1 patient (C. S.) developed a nongrowing leukemia after 3 courses of TRAP but died 3

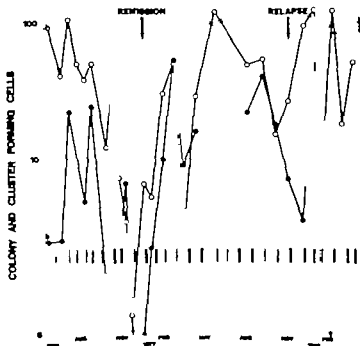


Fig. 2. Serial studies on 46-year-old male (S. S.) with AML. The data are given as colony formation (●) and cluster formation ($\times 10^{-1}$) (○) per µl bone marrow. Chemotherapy courses are shown, TRAP (▣) and COAP (■). The karyotype of marrow cells was normal.

used as maintenance therapy Complete remission was defined as normal bone marrow cellularity with less than 5% blasts when the neutrophil counts had risen to 1,500/μl or greater and the platelet counts to at least 100,000/μl blood

Collection of Bone Marrow

2–3 ml of bone marrow was aspirated from sternum prior to each chemotherapy course or more frequently and collected in 3 ml of McCoy's medium, 100 IU of heparin and 75 U of Varidase (Lederle Laboratories, Pearl River N Y) Marrow particles were disrupted by repeated suction through a needle. Nucleated cells were counted in a Bürker chamber for determination of marrow cellularity

Cell Separation

7 ml of marrow suspended in culture medium were layered on top of 5 ml Isopaque-Ficoll [1] solution in 16-ml plastic tubes. The tubes were centrifuged with a swing out rotor for 10 min at 70 g and for 15 min at 700 g The cells collected on top of the Isopaque Ficoll (A cells) were removed, washed three times in culture medium and used for agar culture. A cells from normal per-

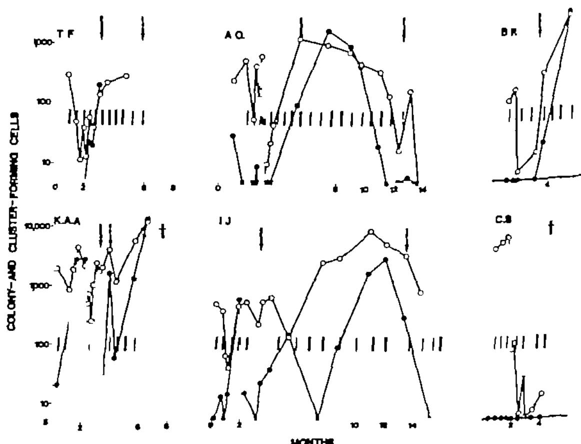


Fig. 1 Serial studies in 6 patients with AML (K.A.A., 24-year-old male; I.J 40-year-old female; C.S 41 year-old female T F 26-year-old male A.O 41 year-old male; B.P 44-year-old male) of colony forming cells (●) per 10⁵ a d cluster-forming cells (O) per 10⁵ seeded marrow cells. The number of cell aggregates grown is given on a logarithmic scale The abscissa shows the time in months after diagnosis. Chemotherapy courses are shown TRAP (▨) and COAP (■) Complete remission (↓) (that of K. A. A. was, however only a partial remission) and relapse (are indicated. The karyotype of marrow cells from C. S. was 45 XO (8.21) and that of T F showed Ph¹-chromosome t (9:22)- the karyotype of the other patients was normal

in relative numbers do not always coincide with variations of absolute numbers of cluster and colony forming cells.

In L. S. (fig. 3) serial analyses were carried out during long periods of the first and second remission phases. Fluctuations of the colony and cluster-forming capacity were prominent with a periodicity of approximately 2–3 months, and persisted when no maintenance therapy was given. That wide fluctuations of the *in vitro* growth pattern can occur during remission is also demonstrated in figures 1 and 2.

The growth pattern of relapse was variable. It was usually associated with disappearance of colony formation, but sometimes with persisting fluctuations of the cluster formation. Independent of the growth pattern during relapse this stage was preceded by decreasing colony and cluster forming capacity sometimes to very low levels (A. O in figure 1 S. S in figure 2, L. S in figure 3 at first and second relapse).

Discussion

The finding of this work is that chemotherapy induces striking oscillations of colony and cluster formation in AML both during induction of remission and remission. The interpretation of our data is complicated because agar culture does not easily discriminate between normal and leukemic colony- and cluster forming cells. However the initial growth pattern was characteristically leukemic in all patients studied

We assume that oscillations of the number of colony and cluster-forming cells are due to recruitment into active proliferative phase of resting leukemic and normal cells upon chemotherapeutic perturbation. It is possible that leukemic blasts somehow inhibit normal clonogenic cells. In fact extracts and conditioned medium of cells from patients with acute leukemia exhibit inhibitory activity on colony formation of normal CFU-C [3] Chemotherapy may provide the necessary decrease of blast cells to allow recruitment of normal granulopoietic precursors and periodical return of normal colony growth. The oscillations depict the struggle between leukemic and normal cells and can all be regarded as potential remissions.

Remission often seems to occur after a period of minimal growth but a prolonged period of minimal or no growth *in vitro* may be a bad prognostic feature as drug-induced aplasia is reported to be prolonged and fatal without early return of colony formation [15]

An intriguing question is whether the myeloblast population in AML requires influx from another compartment to account for its expansion. The cells which grow in agar could be particularly sensitive to chemotherapy as they have a high fraction in S-phase during induction of remission [5] Their eradication may lead to a decrease of leukemic myeloblasts and hypothetically to recruitment of normal precursors and eventually remission.

Striking fluctuations of the colony and cluster incidence were observed also during prolonged remission in accordance with previous reports [11 16] The periodicity is increased during this phase as compared to the period of induction of remission, which could be due to the maintenance therapy consisting of chemotherapy administered with longer intervals. Surprisingly however striking fluctuations also occurred when no maintenance was given. The observation that relapse seems to occur at the initiation

months later without achieving remission. Several patients demonstrate more or less regular fluctuations of the growth pattern during induction of remission. Colony and cluster formation of K.A.A. oscillated in a parallel fashion most of the time. The oscillations showed some correlation with the courses of chemotherapy given since both peaks and troughs usually coincided with the 5-day chemotherapy. However an oscillation was also seen in this patient during month 3 when the interval between the TRAP courses was prolonged due to a severe infection. K.A.A. showed an extremely high colony count during relapse.

During the long induction of remission period of the patient depicted in figure 2 pronounced fluctuations of the colony and cluster incidence were found with peaks approximately every 40–50 days. In this figure results are expressed per microliter marrow and the number of colony and cluster forming cells are seen to decrease in an oscillating fashion during induction of remission. A similar pattern was obvious when the results were expressed as relative numbers per seeded marrow cells (data not shown). However during the relapse period some discrepancies are present since peaks and troughs do not correspond completely when data given as relative and absolute numbers are compared. This indicates that variations

Fig. 3. Serial studies on a 44-year-old male (L.S.) with AML of colony forming cells (●) per 10^5 and cluster forming cells (O) per 10^5 seeded marrow cells. Data for myeloblasts of marrow are also given. Complete remission (↓) and relapse ( ) are indicated as well as courses of chemotherapy: TRAP (▨) and COAP (■).

Acta haemat. 62: 247–250 (1979)

Transformation in Chronic Granulocytic Leukaemia

Different Blast Cell Clones in Different Anatomical Sites[1]

J. A. Vilpo, P. Klemi, O. Lassila, J. Schröder and A. de la Chapelle

Department of Clinical Haematology and Department of Pathology, Turku University Central Hospital, and Department of Medical Microbiology, University of Turku, Turku; Folkhälsan Institute of Genetics, and Department of Medical Genetics, University of Helsinki, Helsinki

Key Words. Blast crisis · Chronic granulocytic leukaemia · Clonal proliferation

Abstract. A 45-year-old female developed blastic metamorphosis in chronic granulocytic leukaemia after 52 months of chronic phase. During the subsequent 6–7 months, lymphosarcomatous enlargements of various lymph nodes developed. The blast cells in lymph nodes differed morphologically from those in bone marrow and blood, being 'lymphoid' non-B non-T non-ALL cells. The karyotype of all metaphases from one lymph node was 47,XX, +21(Ph +) being identical to the karyotype of medullary cells. However, the karyotype of all blasts from another lymph node was 47,XX,+mar(Ph +). It is likely that the local micro-environment controlled the clonal differentiation of these subpopulations which had originated from the same Ph¹-positive multipotent stem cell. In lymph nodes and other extramedullary sites blasts were primitive without differentiation, but a myeloid differentiation in the bone marrow was demonstrated morphologically and cytochemically.

The clinical presentation of chronic granulocytic leukaemia (CGL) is variable. In a majority of cases blastic transformation develops [6]. We present here an unusual case of CGL where cytogenetically and morphologically different blast cell populations occupied anatomically distinctive regions suggesting a malignant clonal proliferation of several subclasses of Philadelphia chromosome positive (Ph +) stem cells.

[1] This work was supported by a grant from the Emil Aaltonen Foundation, Tampere, Finland.

Case Report

In April, 1973, a 45-year-old female was diagnosed to have CGL. The chronic phase of the disease was treated with busulfan or hydroxyurea for 52 months, whereafter the crisis was manifested as myeloid blastosis in bone marrow and peripheral blood. During the subsequent 6–7 months, lymphosarcomatous enlargements of various lymph nodes developed. The clinical situation deteriorated gradually and death occurred on May 11, 1978.

Postmortem examination revealed lymph nodes varying from 1 to 5 cm in diameter in col-

phase of an oscillation could be of importance. Oscillations during remission with or without maintenance therapy may depend on competition between normal and leukemic cells, where the outcome depends on proliferative advantages of the normal or leukemic cell population.

Acknowledgements

We wish to acknowledge with thanks the technical assistance of *Hrefna Sigurdardottir*. This work was supported by the Swedish Cancer Society, Medical Faculty of Lund and Magnus Bergvall's Foundation.

References

1 Bøyum, A. Separation of leukocytes from blood and bone marrow. Scand. J. clin. Lab Invest. *21*: suppl. 97 (1968).
2 Bradley, T. R. and Metcalf, D. The growth of mouse bone marrow cells *in vitro*. Aust. J. exp. Biol. med. Sci. *44*: 287–299 (1966).
3 Broxmeyer, H. E.; Jacobsen, N., Kurland, J.; Mendelsohn, N. and Moore, M. A. S. *In vitro* suppression of normal granulocytic stem cells by inhibitory activity derived from human leukemia cells. J. natn. Cancer Inst. *60*: 497–511 (1978).
4 Greenberg, P. L.; Nichols, W. C., and Schrier, S. L. Granulopoiesis in acute myeloid leukemia and preleukemia. New Engl. J. Med. *184*: 1225–1232 (1971).
5 Gustavsson, A., Olofsson, T. and Olsson, I. Thymidine suiciding of clonogenic cells and myeloblasts in acute myeloid leukemia (to be published).
6 Hayhoe, F. C. J. and Cawley, J. C. Acute leukemia: cellular morphology, cytochemistry and fine structure. Clinics in haematology (Saunders, Philadelphia 1972).
7 Ichikawa, Y.; Pluznik, D. H., and Sachs, L. *In vitro* control of the development of macrophage and granulocyte colonies. Proc. natn. Acad. Sci. USA *56*: 488–495 (1966).
8 Iscove, N. N., Sen, J. S., Till, J. E., and McCulloch, E. A. Colony formation by normal and leukemic human marrow cells in culture: effect of conditioned medium from human leukocytes. Blood *37*: 1–5 (1971).
9 Moore, M. A. S.; Williams, N., and Metcalf, D. *In vitro* colony formation by normal and leukemic human hematopoietic cells: interaction between colony forming and colony stimulating cells. J. natn. Cancer Inst. *50*: 591–602 (1973).
10 Moore, M. A. S.; Spitzer, G.; Williams, N., and Buckley, T. Agar culture studies in 127 cases of untreated acute leukemia: the prognostic value of reclassification of leukemia according to *in vitro* growth characteristics. Blood *44*: 1–18 (1974).
11 Moore, M. A. S. Prediction of relapse and remission in AML by marrow culture criteria. Blood Cells *2*: 109–124 (1976).
12 Olofsson, T. and Olsson, L. Granulopoiesis in chronic myeloid leukemia. II. Serial cloning of blood and bone marrow cells in agar culture. Blood *48*: 351–360 (1976).
13 Paran, M., Sachs, L., Barak, Y. and Resnitzky, P. *In vitro* induction of granulocyte differentiation in hematopoietic cells from leukemic and non-leukemic patients. Proc. natn. Acad. Sci. USA *67*: 1542–1549 (1970).
14 Pike, B. L. and Robinson, W. A. Human bone marrow colony growth in agar-gel. J. cell Physiol. *76*: 77–84 (1970).
15 Spitzer, G., Dicke, K. A., McCredie, K. B., and Barlogie, B. The early detection of remission in acute myelogenous leukemia by *in vitro* cultures. Br. J. Haemat. *35*: 411–418 (1977).
16 Vincent, P. C.; Sutherland, R.; Bradley, M.; Lind, D. and Gunz, F. W. Marrow culture studies in adult acute leukemia at presentation and during remission. Blood *49*: 903–912 (1977).

Received: March 16, 1979
Accepted: June 6, 1979

Inge Olsson, MD, Research Department 2, E-blocket, University Hospital, S-221 85 Lund (Sweden)

Acta haemat. 62, 247–250 (1979)

Transformation in Chronic Granulocytic Leukaemia

Different Blast Cell Clones in Different Anatomical Sites[1]

J. A. Vilpo, P. Klemi, O. Lassila, J. Schröder and A. de la Chapelle

Department of Clinical Haematology and Department of Pathology, Turku University Central Hospital, and Department of Medical Microbiology, University of Turku, Turku; Folkhälsan Institute of Genetics, and Department of Medical Genetics, University of Helsinki, Helsinki

Key Words. Blast crisis · Chronic granulocytic leukaemia · Clonal proliferation

Abstract. A 45-year-old female developed blastic metamorphosis in chronic granulocytic leukaemia after 52 months of chronic phase. During the subsequent 6–7 months, lymphosarcomatous enlargements of various lymph nodes developed. The blast cells in lymph nodes differed morphologically from those in bone marrow and blood, being 'lymphoid' non-B non-T non-ALL cells. The karyotype of all metaphases from one lymph node was 47,XX, +21(Ph +) being identical to the karyotype of medullary cells. However, the karyotype of all blasts from another lymph node was 47,XX,+mar(Ph +). It is likely that the local micro-environment controlled the clonal differentiation of these subpopulations which had originated from the same Ph -positive multipotent stem cell. In lymph nodes and other extramedullary sites blasts were primitive without differentiation, but a myeloid differentiation in the bone marrow was demonstrated morphologically and cytochemically.

The clinical presentation of chronic granulocytic leukaemia (CGL) is variable. In a majority of cases blastic transformation develops [6]. We present here an unusual case of CGL where cytogenetically and morphologically different blast cell populations occupied anatomically distinctive regions suggesting a malignant clonal proliferation of several subclasses of Philadelphia chromosome positive (Ph +) stem cells.

[1] This work was supported by a grant from the Emil Aaltonen Foundation, Tampere, Finland.

Case Report

In April, 1973, a 45-year-old female was diagnosed to have CGL. The chronic phase of the disease was treated with busulfan or hydroxyurea for 52 months, whereafter the crisis was manifested as myeloid blastosis in bone marrow and peripheral blood. During the subsequent 6–7 months, lymphosarcomatous enlargements of various lymph nodes developed. The clinical situation deteriorated gradually and death occurred on May 11 1978.

Postmortem examination revealed lymph nodes varying from 1 to 5 cm in diameter in col-

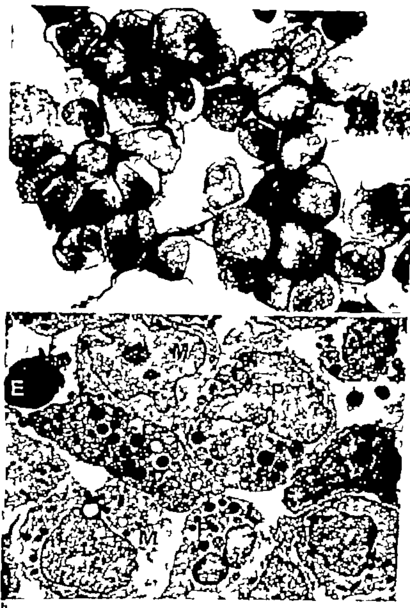


Fig. 1. A Photomicrograph of bone marrow cells. The majority of cells are myeloblasts and promyelocytes. 43% of marrow cells were Sudan black B positive. Most of the blasts belonged to this category May-Grünwald-Giemsa. × 720

B Electron micrograph of bone marrow cells. Myeloblasts (M), promyelocytes (P), and erythroblasts (E) can be identified. Uranyl acetate and lead citrate. × 3,300

lar para-aortal axillary and inguinal regions, and in pulmonary hili as well. In microscopy there were undifferentiated blast cell infiltrations accompanied by marked eosinophilia in enlarged lymph nodes, in pulmonary alveoli renal parenchyma, periportal areas of the liver In the fat tissue surrounding adrenals, and in meninges, too. In the bone marrow the number of blasts was higher than normal, but there were plenty of differentiated granulopoietic megakaryopoietic, and erythropoietic cells to be seen.

Cytological Studies

Bone marrow mitoses were studied on April 4 1978 Every cell (30/30) had a Philadelphia chromosome which had arisen as a result of the standard 9 22 translocation. In addition, there was an extra chromosome No 21 in all bone marrow cells. The karyotype was thus 47,XX,+21(Ph¹+). Most of the bone marrow blasts were Sudan

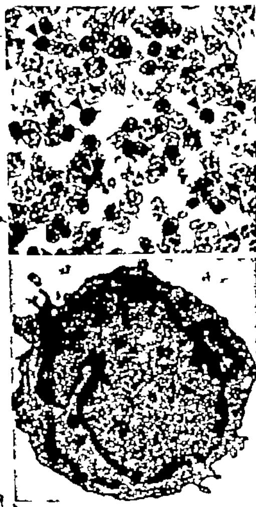


Fig. 2. A Histologic section of lymph node showing massive infiltrations of blasts without any evidence of cytoplasmic differentiation. All blasts were Sudan black B negative. Plenty of mitoses (arrows). HE × 720

B Electron micrograph of primitive blast from fine needle aspiration sample of lymph node. × 8,400

black B positive (fig. 1). Mitoses from 1-day cultures of cells from the peripheral blood without mitogen had an identical karyotype to the bone marrow cells (20/20) 3-day cultures with PHA yielded normal mitoses (46,XX) without the Ph chromosome indicating that the patient s constitutional karyotype was normal.

Cells obtained from a collar lymph node (April 4 1978) had a different karyotype (fig. 2). They also had a Ph chromosome and 47 chromosomes, but the extra chromosome was not a 21 In conventionally stained cells it was indistinguishable from an F chromosome and in G-banded [1] cells it resembled a No. 20 Since it did not appear as metacentric as the normal No 20 chromosomes, it probably represented a morphologically abnormal marker chromosome. This karyotype was, therefore, abbreviated 47,XX,+mar(Ph +).

On May 5 1978 1-day peripheral blood cultures yielded mitoses identical to the previous investigation 47,XX,+21(Ph +). On this occasion another lymph node sample was aspirated. All mitoses obtained from it were 47,XX,+21(Ph¹+). Thus there was no evidence of the marker chromosome seen in the previous biopsy material. The lymph node cells did not express any clear-cut differentiation marker They did not form E or EAC-rosettes. The stainings for SmIg, PAS, acid phosphatase, and α-naphthyl acetate esterase were negative. The lymph node blasts were nonreactive with anti-ALL antiserum (kindly provided by Dr *M F Greaves*).

Discussion

In the two lymph nodes, the presence of the Philadelphia chromosome indicates that

the proliferating cells in both localisations are derived from the same precursor Based on studies of glucose-6-phosphate dehydrogenase heterozygotes [2] as well as on chromosomal evidence [3–5] a unicellular origin of CGL seems likely The later co-existence of cell lines with different supernumerary chromosomes in the present case (21 and mar respectively) indicates the possibility of different events of clonal evolution in different anatomical sites. It remains an open question whether the blast cell populations in bone marrow and in lymph nodes, represent different stages of differentiation and whether they had different capacities to mature. In addition to internal factors, the local micro-environment in the bone marrow may favour the myeloid differentiation of the blast cells. In contrast, the extramedullary blasts of either karyotype did not express any sign of differentiation.

References

1 Chapelle, A de la Vuopio, P and Icén, A. Trisomy in the bone marrow associated with high red cell glutathione reductase activity Blood 47 815–826 (1976).

2 Fialkow P J., Jacobson, R. J and Papayannopoulou, T.. Chronic myelocytic leukemia Clonal origin in a stem cell common to the granulocyte, erythrocyte, platelet and monocyte/macrophage. Am. J Med. 63 125-130 (1977).

3 Hayata, I., Kakati, S., and Sandberg, A. A. On the monoclonal origin of chronic myeloid leukaemia. Proc. Japan Acad. 50: 381-385 (1974).

4 Lawler S. D.. The cytogenetics of chronic leukemia. Clin. Haematol. 6 55–75 (1977)

5 Lawler S D., O'Malley F and Lobb, D. S Chromosome studies in Philadelphia chromosome positive myeloid leukaemia. Scand. J Haematol 17 17–28 (1976).

6 Vallejos, C. S. Trujillo, J M. Cork, A., Bodey G B., McCredie, K. B., and Freireich, E J Blastic crisis in chronic granulocytic leukemia. Experience in 39 patients. Cancer 34 1806–181 (1974).

Received. April 9 1979
Accepted May 3 1979

Dr J A. Vilpo,
Hematology Research Laboratory
Department of Medicine,
Harvard Medical School,
Massachusetts General Hospital,
Boston, MA 02114 (USA)

Acta haemat. 62: 251-256 (1979)

Combination Chemotherapy of Malignant Histiocytosis

E. Palazzo, J. P. Bergerat, J. M. Vetter, A. Bollietot, J. M. Lang and F. Oberling
Clinique des Maladies du Sang and Institut d'Anatomie pathologique, CHU, Strasbourg

Key Words. Combination chemotherapy · Histiocytic medullary reticulosis · Malignant histiocytosis

Abstract. Three patients with malignant histiocytosis treated with combination chemotherapy are reported. Induction treatment included bleomycin, adriamycin, cyclophosphamide, vincristine and prednisone (BACOP). Complete response was obtained in one patient who is alive and well 32 months after diagnosis. A partial response was obtained in the second patient, who is alive and well 35 months after diagnosis. The third patient died with drug-induced agranulocytosis and sepsis.

Introduction

Malignant histiocytosis (MH) is a rare disease characterized by a neoplastic proliferation of abnormal histiocytes [2, 3]. MH was originally described in 1939 by *Scott and Robb-Smith* [10] under the name of 'histiocytic medullary reticulosis'. Until 1966, when *Rappaport* [7] introduced the term malignant histiocytosis, many cases were described under different, although related, names [2]: histiocytic reticulosis, malignant reticuloendotheliosis, réticulose maligne histiocytaire. From the histological point of view MH should be differentiated from benign histiocytic-macrophagic proliferations as well as from histiocytic lymphomas, Hodgkin's disease or more differentiated forms of malignant histiocytic diseases [4, 4, 7, 13]. Criteria for the diagnosis of MH in lymph node biopsies have been set forth by *Byrne and Rappaport* [2]: proliferation of histiocytes, cytologic atypia, phagocytosis, lack of cohesive cell masses, presence of plasma cells and infrequent capsular invasion.

Clinically, MH is characterized by fever, weakness, malaise, hepatomegaly, splenomegaly, lymphadenopathy and jaundice [6, 13]. Although these findings are present in most of the patients, more chronic, usually localized forms, with few systemic symptoms, may be seen [12, 13]. Laboratory studies often show anemia, leukopenia or leukocytosis, thrombocytopenia and hyperbilirubinemia. Untreated patients generally

show a rapid evolution leading to death in 6–12 months.

The purpose of this paper is to report the results of a combination chemotherapy program in three consecutive patients with MH seen at our clinic.

Methods

Three patients with histologically proven MH were eligible for the combination chemotherapy program.

Staging procedures included. physical and routine laboratory examinations, chest x ray IVP upper GI series, small bowel follow through, bilateral pedal lymphography radionuclide scans of liver and spleen, and bone marrow aspiration biopsy When necessary lung tomography bone scan and x ray endoscopies and CNS explorations were performed. After completion of induction therapy patients were reevaluated using the above-mentioned procedures.

Patients were considered in complete remission when there was disappearance of palpable tumor and normalization of previous laboratory x-ray or scan abnormalities attributed to the tumor A partial response indicated more than 50% reduction of the clinical or radiological tumor

The treatment program was as follows: induction therapy (table I) with vincristine, 1 4 mg/m nd adriamycin, 50 mg/m² i v on day 1 cyclophosphamide, 400 mg/m i.v on days 1–5 bleomycin, 4 mg/m i v on days 2 and 5 and prednisone, 100 mg/m orally on days 1–5 After six monthly cycles of chemotherapy the patient was reevaluated. If incomplete remission was obtained, three additional cycles were administered If complete remission was obtained, the patient received CNS prophylaxis with 2,400 rad to the skull and 5 bi weekly injections of intrathecal methotrexate at a dose of 12 mg/m² After hematological recovery the patient was placed for 24 months on maintenance therapy administered every 60 days, rotating the following combinations:

(1) Vincristine, 1.2 mg/m² i.v on day 1 cyclophosphamide, 350 mg/m i.v on days 2 5, and prednisone, 40 mg/m orally on days 1–5.

(2) Adriamycin, 40 mg/m² i.v on day 1 cyclophosphamide, 350 mg/m² i.v on days 2–5 and prednisone, 40 mg/m² on days 1–5.

(3) VM 26, 30 mg/m i v on days 1 5 rubichromomycine, 5 μg/kg i.v on days 1 and 5, and prednisone, 40 mg/m² on days 1–5

(4) Bleomycin 10 mg/m i.v on days 1 and 5; thio-tepa, 5 mg/m i.v on days 1–5, and prednisone, 40 mg/m on days 1–5

Table I. Induction therapy monthly cycle

Drugs	Dose mg/m²	Route	Days 1	2	3	4	5
Vincristine	1 4	i.v					
Adriamycin	50	i v					
Cyclophosphamide	400	i.v					
Bleomycin	4	i.v					
Prednisone	100	oral					

Case Report

Case 1

A 51 year-old man was admitted complaining of fever weight loss, sweating and lymph node swelling. Symptoms had begun 2 months prior to this admission. Physical examination revealed abnormal cervical, axillary and inguinal lymph nodes. Liver and spleen were within normal limits. The chest x-ray and mediastinum tomograms showed enlarged hilar adenopathies. RBC, 3 8 × 10 /liter Hb 1_2/dl WBC, 12.2 × 10⁹ l ter with 57% eosinophils platelets, 189 × 10⁹ liter and ERS, 19 mm in 1 h. Bone marrow aspiration showed eosinophilia and the presence of numerous (29 6%) malignant histiocytes regularly spread on the smears these histiocytes displayed particular features including large size, basophilic cytoplasm, distorted nucleus with reticulated chromatin and an occasional nucleolus. Histological examination of a biopsy specimen showed bone marrow hyperplasia with eosinophilia and marked infiltration by abnormal histiocytes no figure of phagocytosis of erythrocyte, platelet or granulocyt wa observed in bone marrow A cervical lymph node biopsy was performed, supporting the diagnosis of MH (fig. 1)

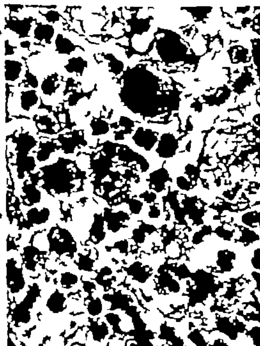


Fig. 1. Case 1. Lymph node biopsy. Frequent atypical histiocytes and eosinophils. HE. 1,150

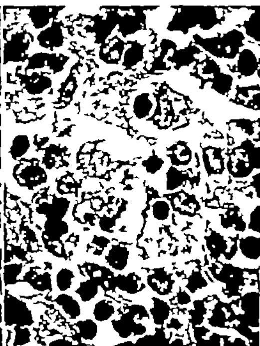


Fig. 2. Case 3. Lymph node biopsy. Abnormal histiocytes showing phagocytosis. HE. × 1,150

Case 2

A 15-year-old girl was admitted with acute dyspnea of sudden onset with clinical and radiological evidence of right-lung atelectasis. No adenopathies, abdominal masses or hepatosplenomegaly were present. RBC, 5.1 10 liter. Hb, 14.5 g/dl, WBC, 9.7 × 10⁹/liter with normal differential count, platelets, 570 × 10⁹/liter and ESR, 68 mm in 1 h. A bronchoscopy showed vegetant tumor in the right main bronchus, whose histology revealed MH. No other clinical or radiological evidence of disease was found.

Case 3

A 28-year-old man admitted with history of bilateral axillary lymph node swelling for 2 months prior to admission. No general symptoms were present. Physical examination was negative except for the axillary nodes. Laboratory data including bone marrow examination were normal. A bilateral pedal lymphography showed abnormal retroperitoneal nodes. An axillary lymph node biopsy supported the diagnostic of MH (fig. 2).

Results

Follow-up and results of chemotherapy are reported in table II. Complete clinical remission was obtained in patient 1 after three courses of BACOP: bone marrow examination performed after the second course showed complete disappearance of the malignant histiocytic infiltration; despite an acceptable hematologic status at the beginning of the third cycle, the patient presented with profond neutropenia (350 PMN/mm³) after the chemotherapy and developed fever: complete hematological recovery oc

Table II. Results and follow-up

Case No.	Response	Remission duration months	Survival months	Toxicity
1	complete	–	5	alopecia agranulocytosis, sepsis
	complete	26 +	32 +	alopecia anemia neutropenia
3	partial	26 +	35 +	alopecia polyneuritis diminution of lung vital capacity

Remission duration refers to the end of induction therapy and survival to the time of diagnosis.

curred a few days later (7 000 PMN/mm³) but the patient died in septic condition in spite of antibiotic treatment he was treated outside the clinic and more information is not available No etiologic agent was isolated and no autopsy was performed.

Case 2 showed dramatic subjective and radiological improvement after the first cycle of BACOP After six cycles, a new bronchoscopy showed a normal mucosa and negative biopsies. The patient is alive and without evidence of disease 26 months after completing induction therapy and 32 months after diagnosis. In case 3 a partial remission was obtained after nine cycles of BACOP with persistence of a small axillary node and slightly abnormal lymphography The treatment was completed with radiotherapy to the axilla and retroperitoneal area. He is alive and well 35 months after diagnosis.

Toxic effects included alopecia and loss of deep-tendon reflexes in all patients. Case 1 developed fatal agranulocytosis. Case 2 neutropenia and slight anemia. Patient 3 had vincristine-induced polyneuritis with parestesias and EMG abnormalities, and diminution of pulmonary vital capacity due to bleomycin. In spite of these toxic effects, more than 80% of the scheduled doses were administrated to all patients.

Discussion

Numerous drugs have been used in MH either alone or in combination [1 6] Most of the reports refer to small series (since MH is a rare disease) and show that combination therapy has yielded the best results. *Alexander and Daniels* [1] report their experience with 16 patients treated with different regimens The best responses were obtained with the combination of cyclophosphamide, adriamycin vincristine and prednisone (CHOP) with 5/7 responses (complete response + partial remission). Median survival for responders was 23 months with 1 patient in unmaintained remission for more than 1 year In 1976,

Stein et al [11] reported a case of systemic MH in a 16-year-old girl who achieved complete remission with COPP and was alive and well 55 months after cessation of therapy *Lamper et al.* [6] reported their results with CHOP therapy in a group of 7 patients. They obtained 4 complete remissions with survivals of 15 18+ 25+ and 31+ months. 3 of these patients were off therapy for 14 18 and 19 months. In 1977 *Rauskanen et al.* [8] reported 3 children with MH treated with chemotherapy 1 of the patients underwent splenectomy was treated with COP and achieved complete remission, lasting for 18+ months Another child received BACOP and died of sepsis after 2 months of treatment. No atypical histiocytes were found at autopsy

BACOP therapy has thus seldom been reported in MH. In lew of the poor results obtained with other therapies and the promising results attained in lymphomas with combinations including adriamycin [5] we started using a combination of bleomycin, adriamycin, cyclophosphamide, vincristine and prednisone in our patients with MH in 1975 In 1976, *Schein et al* [9] reported the results with a similar combination in the treatment of histiocytic lymphomas.

BACOP appears as an effective therapy for this disease Dramatic remission was obtained in all patients with a disease-free interval of more than 32 months for case 2 and of more than 35 months for case 3 who was also treated by radiotherapy for small residual nodes.

Patient 1 who showed clinical evidence of remission, exhibited transient neutropenia after completing the third cycle of BA COP and died of sepsis It is difficult to evaluate the toxicity of the BACOP protocol on this restricted number of MH. However the same combination chemotherapy regimen was used in our institution since 1975 for more than 50 patients under the age of 65 with disseminated non-Hodgkin lymphomas of unfavorable histology preliminary results of this study were published elsewhere Profound post-chemotherapeutic neutropenia occurred in 10% of the cases, neutropenia was usually promptly reversible and severe septic complications were rare. Cardiac accidents due to adriamycin were a oided with maximum cumulative doses of 500 mg/m and systematic echocardiographic monitoring Repeated administration of bleomycin were well tolerated in this chemotherapeutic regimen, probably because of the concomitant corticotherapy and no case of acute intolerance or chronic wasting disease was recorded, severe pulmonary fibrosis was rare, due to the low cumulative doses of bleomycin scheduled in this protocol. Thus the toxicity of the BACOP regimen is acceptable under appropriate monitoring.

We conclude that this chemotherapy regimen, as applied by us in MH, is effective for this rare disease, and we feel that its usefulness and effectivity are comparable to those of CHOP and COPP

References

1 Alexander M. and Daniels, J R. Chemotherapy of malignant histiocytosis in adults. Cancer N Y 39 1011–1017 (1977).

2 Byrne, G E and Rappaport, H. Malignant histiocytosis, in Akazaki, Rappaport, Berard, Bennett and Ishikawa malignant diseases of the hematopoietic system, pp 145–162 (University Park Press, Tokyo 1973).

3 Chou, M J and Golde, D W A review and reevaluation of the histiocytic disorders. Am. J Med 55, 49–60 (1973)

4 Dorfman, R. F and Warnke, R. Lymphoid-

enopathy simulating the malignant lymphomas. Human Pathol. *5* 519–550 (1974)

5 Gottlieb, J A. Gutterman, J U., McCredie, K. B Rodriguez, V and Frei, E., III Chemotherapy of malignant lymphoma with adriamycin. Cancer Res. *33* 3024–3028 (1973)

6 Lampert, I A. Catovsky D and Bergier N., Malignant histiocytosis. a clinico-pathological study of 12 cases. Br J Haemat. *40* 65–77 (1978)

7 Rappaport, H Tumors of the hematopoietic system. Atlas of tumor pathology pp 49–63 (Armed Forces Institute of Pathology Washington 1966).

8 Ruuskanen, O., Kero P Rajamäki, A., Ekfors, T Vilkki, P and Nordman, E. Malignant histiocytosis. Acta pediat. scand. *66* 249–254 (1977)

9 Schein, P S. DeVita, V T Hubbard, S. Chabner B A., Canellos, G P., Berard, C., and Young, R. C. Bleomycin, adriamycin, cyclophosphamide, vincristine and prednisone (BACOP) combination chemotherapy in the treatment of advanced diffuse histiocytic lymphoma. Ann. intern. Med. *85* 417–422 (1976)

10 Scott, R. B. and Robb-Smith, A. H. T Histiocytic medullary reticulosis. Lancet *ii.* 194–198 (1939).

11 Stein, R. S. Moran, E. M., and Byrne, G. E Malignant histiocytosis: complete remission with combination chemotherapy Cancer N. Y *38* 1083–1086 (1976)

12 Vardiman, J W Byrne, G E., and Rappaport, H., Malignant histiocytosis with massive splenomegaly in asymptomatic patients. Cancer N Y *36* 419–427 (1975).

13 Warnke, R. A. Kim, H., and Dorfman, R. F Malignant histiocytosis (Histiocytic medullary reticulosis). Cancer N Y *35* 215–230 (1975).

Received. January 9 1979
Accepted. July 6, 1979

Prof F Oberling,
Clinique des Maladies du Sang,
Hôpital Civil, F–67000 Strasbourg,
(France)

Acta haemat. 62 257–261 (1979)

Multiple Myeloma without Detectable Ig Synthesis

Anna Maria Ferraris Enrico Haupt and Massimo Ratti

Department of Haematology University of Genova, Genova, and
Department of Medicine, Lavagna Hospital, Lavagna

Key Words. Immunofluorescence Myeloma Nonproductor Plasma cells

Abstract. A case of nonproductor myeloma is reported. The diagnosis was supported by the radiological findings, the heavy marrow infiltration of malignant plasma cells, the absence of a monoclonal component in the serum or urine and the failure to demonstrate intracytoplasmic immunoglobulins with immunofluorescent techniques. The clinical findings of our patient are similar to those reported for the 5 cases of nonproductor myeloma described so far indicating that there are no characteristic features differentiating nonproductor myeloma from productor myelomas.

Introduction

Multiple myeloma is a malignant disease characterized by a monoclonal proliferation of plasma cells which results in the production of large amounts of homogenous M-type immunoglobulin [1 10 13] In about 1 / of cases the malignant plasma cells fail to secret Ig, and no monoclonal component is detectable by electrophoresis or immunochemical procedures in the serum and/or urine [2, 9] In the large majority of these cases immunofluorescent techniques demonstrate the presence of immunoglobulin in the cytoplasm of the malignant plasma cells [1 6, 12, 16] Accordingly these forms are classified as 'nonsecretory myelomas which implies a block in the process of secretion of the monoclonal protein. In the past years a very small number of cases have been reported in which not only the M-component was absent from the serum, but also no immunoglobulin was detectable in the cytoplasm of the malignant plasma cells [5 7 11 15 17] We report here what we believe to be the sixth case so far described.

Case Report

A 55-year-old woman was admitted to the hospital because of pathological costal fracture. She was well until 3 years before when she began to experience mild diffuse bone pain. On admission the physical examination showed no lymphadenopathy or hepatosplenomegaly there was generalized bone tenderness.

Skeletal X-rays showed disseminated 'punched-out' lesions in the skull (fig. 1), clavicles, ribs

enopathy simulating the malignant lymphomas. Human Pathol. *5* 519–550 (1974).

5 Gottlieb, J A., Gutterman, J U McCredie, K. B., Rodriguez, V and Frei, E., III Chemotherapy of malignant lymphoma with adriamycin. Cancer Res. *33* 3024–3028 (1973)

6 Lampert, I A., Catovsky D and Bergier N Malignant histiocytosis. a clinico-pathological study of 12 cases. Br J Haemat. *40* 65–77 (1978)

7 Rappaport, H Tumors of the hematopoietic system. Atlas of tumor pathology pp 49–63 (Armed Forces Institute of Pathology Washington 1966).

8 Ruuskanen, O Kero, P Rajamäki, A., Ekfors, T Viikki, P and Nordman, E. Malignant histiocytosis. Acta pediat. scand. *66*. 249–254 (1977).

9 Schein, P S. DeVita, V T., Hubbard, S. Chabner B A. Canellos, G P Berard, C., and Young, R C.. Bleomycin, adriamycin, cyclophosphamide, vincristine and prednisone (BACOP) combination chemotherapy in the treatment of advanced diffuse histiocytic lymphoma. Ann. Intern. Med. *85* 417–422 (1976)

10 Scott, R. B and Robb-Smith, A. H. T Histiocytic medullary reticulosis. Lancet *ii*. 194–198 (1939).

11 Stein, R. S. Moran, E. M., and Byrne, G E. Malignant histiocytosis: complete remission with combination chemotherapy Cancer N Y *38* 1083–1086 (1976).

12 Vardiman, J W Byrne, G. E., and Rappaport, H. Malignant histiocytosis with massive splenomegaly in asymptomatic patients. Cancer N Y *36* 419–427 (1975).

13 Warnke, R. A. Kim, H., and Dorfman, R. F Malignant histiocytosis (Histiocytic medullary reticulosis). Cancer N Y *35* 215–230 (1975)

Received January 9 1979
Accepted July 6, 1979

Prof F Oberling,
Clinique des Maladies du Sang,
Hôpital Civil, F–67000 Strasbourg,
(France)

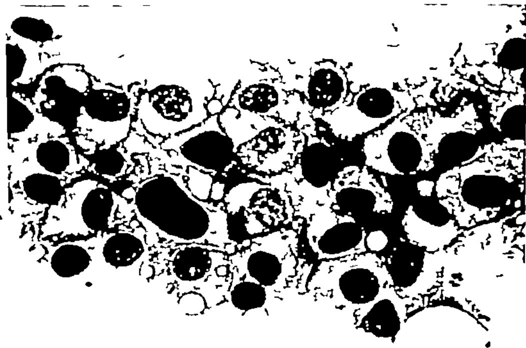


Fig. 2. Photomicrograph of bone marrow aspirate showing heavy infiltration of malignant plasma cell 1,000.

conditions rapidly improved Repeated X-ray examinations failed to demonstrate an objective regression of the preexisting bone lesions The patient is now free from bone pain and is leading completely normal life

Discussion

The clinical, radiological and histological picture of our patient was consistent with a diagnosis of multiple myeloma, and the absence of a monoclonal immunoglobulin in the serum and urine indicated that it was one of the rare cases of nonsecretory myeloma [1 6, 9 1] The immunofluorescent studies performed on the bone marrow specimens failed to demonstrate the presence of plasma cells containing in their cytoplasm a monoclonal immunoglobulin demonstrating that we were dealing with an even rarer case of nonproductor myeloma [5 7 11 15 17] The histological, laboratory and immunological features of our patient are similar to those reported for the 5 cases of nonproductor myeloma described in the literature so far [5 7 11 15 17]

Our findings lend further evidence to the hypothesis that there are no characteristic features differentiating nonproductor myelomas from nonsecretory myelomas [11], apart from the presence of the monoclonal component in the serum and or urine, with its clinical consequences (renal failure, amyloidosis, hyperviscosity syndrome). However



Fig. 1 Skull roentgenogram showing multiple osteolytic lesions.

and sternum. The erythrocyte sedimentatio rate was 42 mm/1 h, the PCV 0.39 the Hb 12 g/dl, the white cell count was 5 600 × 10 ⁹/l, with a normal differential count. Liver function tests, electrolytes, serum creatinine and creatinine clearance were all normal. The alkaline phosphatase was raised (130 mU/ml normal range 30–50 mU/ml) Urinalysis was negative for protein.

The total serum protein was 7 4 g/100 ml. Serum electrophoresis showed that the albumin was 57 1 /ₒ, the α-globulin 6 8%, the α_2-globuli 11 4 /ₒ, the β-globulin 12.1 /ₒ, and the γ-globulin 12.5%/ Immunoglobulin concentration determined by radial immunodiffusion was. IgG 759 mg%, IgA 87 mg%/ and IgM 22 mg%. Immunoelectrophoresis with a polyvalent antiserum as well as anti k, λ, IgG IgA, IgM, IgE, IgD antisera did not reveal any monoclonal component Bence Jones protein was not detected even in concentrated urine. Urine immunoelectrophoresis with an antilight chain (K and λ type) antiserum was also negative.

A bone marrow aspirate obtained from the posterior iliac crest showed normal erythroblastic, granuloblastic and megakaryoblastic series and an increase in the number of plasma cells (up to 20%/) which exhibited nuclear-cytoplasmic abnormal ties. A sampl of the bone marrow aspirate was smeared using a cytocentrifuge, fixed and stained with a polyvalent antiserum or with an anti-L chain antiserum conjugated with fluorescein isothiocyanate (FITC) The antihuman F (ab)₂ and λ light chain antisera were raised in rabbits or goats, made specific by absorption with proteins coupled to Sepharose 4-B and tested for specificity as detailed elsewhere [3, 4] IgG fractions were prepared from the antisera and conjugated with FITC as previously reported [4]. Less than 1 % Ig containing cells, with the morphology of plasmoblasts or mature plasma cells, were seen.

An open biopsy of the left clavicle (where a confluent osteolytic area was seen on the X-rays) showed a massive infiltration of malignant appearing pl sma cells (Fig. 2). The patient was started on a course of melphalan 10 mg day and prednisone 50 mg day for 10 d ys Although she suffered from a pathological fracture of the right clavicle on the 2nd day of treatment, her general

15 River G L., Tewksbury D. A., and Fudenberg, H. H 'Nonsecretory' multiple myeloma. Blood 40 204 (1972).
16 Ryckewaert, A Kuntz, D Bonhomme, J Preud'Homme, J L., Hurez, D et Seligmann, M., U cas de myélome non sécrétant vec déminéralisation vertébrale diffuse et rétention intracellulaire de la globuline myelomateuse Revue, Rhum. Mal. ostéo-artic. 11 621 (1969)
17 Stavem, P Froland, S. S., Haugen, H. F and Lislerud, A Scand. J Haematol 17 89 (1976).
18 Waldmann, T A. and Broder S Suppressor cell in the regulation of the immune response. Prog. clin. Immunol. 3 155 (1977).

Received March 30 1979
Accepted. May 18, 1979

Anna Maria Ferraris, Ematologia ISMI,
Viale Benedetto XV 6, I-16132 Genova (Italy)

er in our patient the bone lesions have a very peculiar distribution being confined to the upper half of the body

The constant finding of depressed levels of normal serum immunoglobulin is not characteristic of nonproductor myelomas [11 15] since it is also a common feature of most myeloma cases [8 14] This fact has been explained by the heavy infiltration of the bone marrow which is the predominant site of Ig synthesis [8 13] Moreover an increased number of suppressor T cells has been demonstrated in multiple myeloma [18]

The phenomenon of nonproduction in multiple myeloma remains speculative As to the possible cause of the emergence and proliferation of an atypical clone of plasma cells without demonstrable immunoglobulin in the cytoplasm three possible mechanisms can be envisaged. (1) the cell synthesizes an abnormal immunoglobulin which is as rapidly degraded within the cytoplasm (2) the gene coding for the synthesis of the immunoglobulin is mutated in such a way that its product is no more recognizable as an immunoglobulin (3) the immunoglobulin gene is functionally defective

Acknowledgement

This work has been supported in part by CNR grants No 41/77 01566.65 and No. 9/78.001186.

We thank Dr *G Corte* for the gift of the FITC conjugated antisera a d assistance in staining the smears, and Dr *M Ferrarini* for advice and criticism.

References

1 Arend, W P and Adamson, J W Non-secretory myeloma-immunofluorescent demonstration of paraprotein within bone marrow plasma cells. Cancer *33* 721 (1974).

2 Di Guglielmo, R.. Unusual morphologic and humoral conditions in the field of plasmocytomas and M-type dysproteinemia. Acta med scand. *445* suppl., p 260 (1966).

3 Ferrarini, M. Bargellesi, A., Corte, G., Viale, G and Pernis, B Comparative studies of membrane and cytoplasmic immunoglobulin classes in human lymphoid cells. Ann. N Y Acad. Sci *254* 243 (1975).

4 Ferrarini, M., Corte, G Viale, G., Durante, M L., and Bargellesi, A.. Membrane Ig on human lymphocytes, rate of turnover of IgD and IgM on the surface of human tonsil cells. Eur. J Immunol. *6* 372 (1976).

5 Gach, J Simar L., and Salmon, J Multiple myeloma without M-type proteinemia. Report of a case with immunologic and ultrastructure studies. Am. J Med. *50* 835 (1971).

6 Hurez, D., Preud'Homme, J L., and Seligman, M.. Intracellular 'monoclonal' immunoglobulin in non secretory human myeloma. J Immun. *104* 263 (1970).

7 Indiveri, F Barabino, A. Santolini, M E., *et al.*. Non-secretory multiple myeloma. Report of a case. Acta haemat. *51* 302 (1974)

8 Isobe, T and Osserman, E. F Pathologic conditions associated with plasma cell dyscrasias: a study of 806 cases. Ann. N Y Acad. Sci. *190* 507 (1971)

9 Kim, I. Harley J B and Wekaler B Multiple myeloma without initial paraprotein. Am. J med. Sci. *264* 267 (1972).

10 Kyle, R. A. Multiple myeloma. review of 869 cases. M yo Clin Proc. *50* 29 (1975).

11 Mancilla, R. and Davis, G L. Nonsecretory multiple myeloma. Immunohistologic and ultrastructural observations on two patients. Am. J Med. *63* 1015 (1977)

12 Menkes, C. J., Herreman, G Preud'Homme, J L., *et al* Myeloma à plasmocytes non excrétants. Nouv Presse méd. *1* 309 (1972).

13 Osserman, E. F and Takatsuki, K.. Plasma cell myeloma-gamma globul'n synthesi and structure. Medicin Baltimore *42* 357 (1963).

14 Osserman, E. F and Fahey J L.. Plasma cell dyscrasias. current clinical and biochemical concepts. Combined Staff Clinic. Am. J Med. *44* 256 (1968)

scheduled to receive MOPP chemotherapy after completion of radiotherapy

The aim of this study was to evaluate the role of adjuvant MOPP chemotherapy after radiotherapy in patients with stage I and II A with respect to increasing survival, relapse rate and the toxicity related to chemotherapy

Patients and Methods

85 patients with biopsy evidence of HD in stage I, II A and B NS were first treated from 1970 to 1976. Stage classification was done according to the Ann Arbor protocol [2] and histologic classification according to *Ry* [9]. Every patient was submitted to lymphangiography bone biopsy laparotomy and splenectomy The patients' distribution according to stage and therapeutic program is reported in table I.

The two following different therapeutic programs were applied (1) radiotherapy alone (upper mantle down to the fifth lumbar vertebra and also splenic hilum labelled with silver clips after splenectomy), and (2) radiotherapy followed by three courses of MOPP 3 months after completion of radiotherapy Radiotherapy was given by 60 Co source using patchwork multiple treatment fields The estimated tumor dose delivered was 4,500 rads in 5–6 weeks.

I recent years, prednisone was eliminated

Table I. Patient distribution according to age, sex, stage and therapeutic program

	Radiotherapy	Radiotherapy and chemotherapy
Patients,	31	54
Age, years	30 (15–56)	29 (14–54)
Sex, M/F	18/13	29 25
Stage		
I A	10	10
I B		
II A	21	25
II B		14
II B		3

from the MOPP regimen as previously described by *D Vita et l.* [4] in patients with prior mediastinal irradiation in order to decrease the risk of cardiopulmonary complications. These complications wer apparently related to the discontinuance of steroids [3]. Chemotherapy was delayed from 1 to 3 weeks in the event of (< 2,500 leucocytes/µl) neutropenia and or thrombocytopenia (< 100,000 platelets l). Once therapy was completed, all patients were examined at regular intervals in the out-patient clinic. Chest radiograms, blood counts, sedimentation rate and liver function tests were routinely performed t every check-up W detected the elapses that occurred through tissue biopsy whenever possible or through radiographic evidence of progressing disease in all other cases (mainly lung and or mediastinal involvement)

T analyze the relapse-free survival curves we applied the method described by *Kaplan and Meier* [8] and the Logrank test according to *Peto et al* [11].

Results

Table II summarizes the results obtained in the 85 patients evaluated and submitted to two therapeutic programs.

Radiotherapy Alone

31 patients with the pathologic stage I and II A NS received radiotherapy alone. All of them are alive, but 9 have relapsed. 10 of these 31 patients were stage I and _1 stage II. Among the 9 relapsed patients only 2 were stage I and 7 stage II They were all given six courses of MOPP and in some cases radiotherapy on the site of the relapse and all but 1 achieved a second complete remission. No patient with stage I A, without mediastinum involvement, relapsed (table III).

Radiotherapy and Chemotherapy

35 patients with stage I and II A and 19 patients with stage I, II and II_E B NS were

Acta haemat 62 262–266 (1979)

Management of Nodular Sclerosis Hodgkin's Disease Stage I, II A and B Evidence for a Beneficial Effect of MOPP on the Relapse Rate[1]

Francesco Lauria Michele Baccarani Lucio Babini Ermanno Emiliani Mauro Fiacchini Marco Gobbi Patrizio Mazza Ruggero Sciascia and Sante Tura

Chair of Hematology and Radium Institute, University of Bologna, School of Medicine, Division of Hematology St. Orsola s University Hospital, Bologna

Key Words. Hodgkin s Disease MOPP chemotherapy Nodular sclerosis Pathologic stage I II A and B

Abstract. 85 patients with previously untreated Hodgkin s disease with stage I II A and B nodular sclerosis were treated 31 of them with stage I and II A were submitted to radiotherapy alone All are alive but 9 of them (30%) relapsed. On the contrary 35 patients with stage I and II A and 19 with stage I II and II_E B were submitted to radiotherapy followed by three courses of MOPP All 54 patients are alive and relapse-free. No severe complication related to chemotherapy was observed. The analysis of results suggests that 3 courses of MOPP can significantly ($p < 0\ 00025$) reduce the relapse rate in patients with stage I and II nodular sclerosis, eligible for radiotherapy without increasing morbidity

Introduction

The high megavoltage radiotherapy for early-stage Hodgkin s disease (HD) can cure most patients with HD stage I and II A lymphocytic predominance (LP) and nodular sclerosis (NS) [5–7 13] However a small number of these patients is at risk of relapse, and recently it has been suggested that chemotherapy (mainly MOPP) after radiotherapy in selected patients, can effectively destroy the small residual foci which are responsible for late relapse after radiotherapy in limited HD [1 7 10 13]

Radiotherapy alone proved to be successful in patients in stage I II A LP· all our 17 patients treated in this way achieved complete remission (CR) and all of them so far have been relapse free for 14–86 months Patients with stage I II A NS did not behave in the same manner as 9 of 31 patients (30%) have relapsed In contrast, no stage I II B NS patient who had been scheduled to receive 3 courses of MOPP chemotherapy after radiotherapy has relapsed as yet. For this reason all subsequent patients with stage I and II A NS were

[1] This work was supported in part by 'Centro Bolognese per lo studio delle leucemie e dei linfomi

scheduled to receive MOPP chemotherapy after completion of radiotherapy

The aim of this study was to evaluate the role of adjuvant MOPP chemotherapy after radiotherapy in patients with stage I and II A with respect to increasing survival, re lapse rate and the toxicity related to chemotherapy

Patients and Methods

85 patients with biopsy evidence of HD in stage I, II A and B NS were first treated from 1970 to 1976. Stage classification was done according to the Ann Arbor protocol [2] and histologic classification according t *Ry* [9]. Every patient was submitted to lymphangiography bone biopsy laparotomy and splenectomy The patients' distribution according to stage and therapeutic program is reported in table I.

The two following different therapeutic programs were applied. (1) radiotherapy alone (upper mantle dow to the fifth lumbar vertebra and also splenic hilum labelled with silver clips fter sple nectomy), and (2) radiotherapy followed by three courses of MOPP 3 months after completion of radiotherapy Radiotherapy was given by 60 Co source using patchwork multiple treatment fields. The estimated tumor dose delivered was 4,500 rads in 5–6 weeks

I recent years, prednisone was eliminated from the MOPP regimen as previously described by *D Vita et al.* [4] in patients with prior mediastinal irradiation in order to decrease the risk of cardiopulmonary complications. These complications were apparently related to the discontinuance of steroids [3]. Chemotherapy was delayed from 1 to 3 weeks in the event of (< ,500 leucocytes/μl) neutropenia and/or thrombocytopenia (< 100,000 platelets/μl). Once therapy was completed, ll patients were examined at regular inter als in the out-patient clinic. Chest radiograms, blood counts, sedimentation rate and liver function tests were routinely performed at every check-up W detected the relapses that occurred through tissue biopsy whenever possible or through radiographic evidence of progressing disease in ll other cases (mainly lung and/or mediastinal involvement).

T analyze the relapse-free survival curves w applied the method described by *Kaplan and Meier* [8] and the Logrank test according to *Peto et al* [11].

Table I. Patient distribution according to age, sex, stage and therapeutic program

	Radiotherapy	Radiotherapy and chemotherapy
Patients,	31	54
Age, years	30 (15–56)	29 (14–54)
Sex, M/F	18 13	29/25
Stage		
I A	10	10
I B		
II A	1	25
II B		14
II_EB		3

Results

Table II summarizes the results obtained in the 85 patients evaluated and submitted to two therapeutic programs.

Radiotherapy Alone

31 patients with the pathologic stage I and II A NS received radiotherapy alone. All of them are alive, but 9 have relapsed. 10 of these 31 patients were stage I and 21 stage II Among the 9 relapsed patients only .. were stage I and 7 stage II. They were all given six courses of MOPP and in some cases radiotherapy on the site of the relapse and all but 1 achieved a second complete remission. No patient with stage I A, without mediastinum involvement, relapsed (table III).

Radiotherapy and Chemotherapy

35 patients with stage I and II A and 19 patients with stage I, II and II_E B NS were

Acta haemat 62. 262–266 (1979)

Management of Nodular Sclerosis Hodgkin's Disease Stage I, II A and B Evidence for a Beneficial Effect of MOPP on the Relapse Rate[1]

Francesco Lauria Michele Baccarani Lucio Babini Ermanno Emiliani Mauro Fiacchini Marco Gobbi Patrizio Mazza Ruggero Sciascia and Sante Tura

Chair of Hematology and Radium Institute, University of Bologna, School of Medicine, Division of Hematology St. Orsola s University Hospital, Bologna

Key Words. Hodgkin s Disease MOPP chemotherapy Nodular sclerosis Pathologic stage I II A and B

Abstract. 85 patients with previously untreated Hodgkin s disease with stage I II A and B nodular sclerosis were treated 31 of them with stage I and II A were submitted to radiotherapy alone All are alive, but 9 of them (30%) relapsed. On the contrary 35 patients with stage I and II A, and 19 with stage I II and II_E B were submitted to radiotherapy followed by three courses of MOPP All 54 patients are alive and relapse-free. No severe complication related to chemotherapy was observed. The analysis of results suggests that 3 courses of MOPP can significantly ($p < 0.00025$) reduce the relapse rate in patients with stage I and II nodular sclerosis, eligible for radiotherapy without increasing morbidity

Introduction

The high megavoltage radiotherapy for early-stage Hodgkin s disease (HD) can cure most patients with HD stage I and II A, lymphocytic predominance (LP) and nodular sclerosis (NS) [5–7 13] However a small number of these patients is at risk of relapse, and recently it has been suggested that chemotherapy (mainly MOPP) after radiotherapy in selected patients, can effectively destroy the small residual foci which are responsible for late relapse after radiotherapy in limited HD [1 7 10 13]

Radiotherapy alone proved to be successful in patients in stage I II A LP all our 17 patients treated in this way achieved complete remission (CR) and all of them so far have been relapse-free for 14–86 months. Patients with stage I II A NS did not behave in the same manner as 9 of 31 patients (30%) have relapsed. In contrast, no stage I II B NS patient who had been scheduled to receive 3 courses of MOPP chemotherapy after radiotherapy has relapsed as yet. For this reason all subsequent patients with stage I and II A NS were

[1] This work was supported in part by 'Centro Bolognese per lo studio delle leucemie e dei linfomi'

scheduled to receive MOPP chemotherapy after completion of radiotherapy

The aim of this study was to evaluate the role of adjuvant MOPP chemotherapy after radiotherapy in patients with stage I and II A with respect to increasing survival re lapse rate and the toxicity related to chemotherapy

Patients and Methods

85 patients with biopsy evidence of HD in stage I, II A and B NS were first treated from 1970 to 1976. Stage classification was done cording to the Ann Arbor protocol [2] and histologic classification according t Ry [9] E ery patient was submitted to lymphangiography bone biopsy laparotomy and splenectomy The patients distribution according to stage and therapeutic program is reported in tabl I.

The tw following different therapeutic programs were applied: (1) radiotherapy alone (upper mantle down to the fifth lumbar vertebra and also splenic hilum labelled with silver clips after splenectomy), and (2) radiotherapy followed by three courses of MOPP 3 months after completion of radiotherapy Radiotherapy was given by 60 Co source using patchwork multiple treatment fields The estimated tumor dose delivered was 4,500 rads in 5–6 weeks.

In recent years, prednisone was eliminated

Table I. Patient distribution according t age, sex, stage and therapeutic program

	Radiotherapy	Radiotherapy and chemotherapy
Patients,	31	54
Age, years	30 (15–56)	29 (14–54)
Sex, M/F	18/13	29 25
Stage		
I A	10	10
I B		2
II A	21	25
II B	–	14
II_EB		3

from the MOPP regimen as previously described by D Vita et al. [4] in patients with prior mediastinal irradiation in order to decrease the risk of cardiopulmonary complications. These complications were pparently related t the discontinuance of steroids [3]. Chemotherapy was delayed from 1 to 3 week in the event of (< 2,500 leucocytes l) neutropenia and or thrombocytopenia (< 100,000 platelets l). Once therapy was completed, all patients wer examined at regular inter als in the out-patient clinic Chest radiograms, blood counts, sedimentation rate and liver function tests ere routinely performed at every check-up. W detected the relapses that occurred through tissue biopsy whenever possibl or through radiographic evidence of progressing disease in all other cases (mainly lung and/or mediastinal involvement).

T analyze the relapse-free survival curves we applied the method described by Kaplan and Meier [8] and the Logrank test according to Peto et al. [11].

Results

Table II summarizes the results obtained in the 85 patients evaluated and submitted to two therapeutic programs.

Radiotherapy Alone

31 patients with the pathologic stage I and II A NS received radiotherapy alone. All of them are alive, but 9 have relapsed. 10 of these 31 patients were stage I and 21 stage II Among the 9 relapsed patients only were stage I and 7 stage II. They were all given six courses of MOPP and in some cases radiotherapy on the site of the relapse and all but 1 achieved a second complete remission. No patient with stage I A without mediastinum involvement, relapsed (table III).

Radiotherapy and Chemotherapy

35 patients with stage I and II A and 19 patients with stage I, II and II_E B NS were

submitted to radiotherapy followed by 3 courses of MOPP. All patients are alive and relapse free. The relapse rate of patients treated with radiotherapy alone is significantly different compared to patients treated with radiotherapy plus 3 courses of MOPP ($p < 0.00025$). Figure 1 shows the relapse-free survival curves according to therapy.

Table II. Analysis of relapses according to stage and therapeutic program

	Radiotherapy	Radiotherapy and chemotherapy
Patients, n	9/31[1] p <0.00025	0/54[1]
Stage		
I A	2/10	0/10
I B	–	0/2
II A	7/21	0/25
II B	–	0/14
II$_E$B	–	0/2

1 Number of patients with relapse/total number of patients.

Analysis of Relapses

The analysis of relapses according to age, stage and site of disease is shown in table III. As mentioned above, no patient submitted to radiotherapy and chemotherapy relapsed. Out of the 31 patients with stage I and II A treated with radiotherapy alone, 2 stage I and 7 stage II relapsed 5, 8, 14, 16, 18, 24, 28, 36 and 60 months, respectively, after the end of radiotherapy. The last one relapsed immediately after pregnancy. 2 of the 10 patients with stage I showed involvement of the mediastinum at diagnosis and both relapsed. The 7 stage II patients with relapse showed involvement of their left supraclavicular nodes and mediastinum at diagnosis. The site of relapse was as follows: 2 cases of contiguous non-irra-

Table III. Analysis of relapses according to sex, age, stage, site of disease and results after second therapy

Case	Sex	Age	Stage	Early involvement	Relapse site	Treatment	Current status and follow-up (months)
T.R.	M	29	IIA	SC, M	I	MOPP + LR	NED (15)
S.M.	M	18	IIA	C, SC, M	SC, A, M	MOPP	NED (28)
D.T.A.	F	22	IIA	SC, M	M, L, P	MOPP	NED (44)
D.M.M.	F	22	IIA	C, SC, A, M	L	MOPP	NED (54)
M.G.	M	21	IIA	C, SC, M	M, L, C, SC	MOPP	NED (35)
A.M.	F	15	IIA	SC, M	IN	MOPP + LR	NED (34)
L.G.	F	34	IIA	SC, M	IC	MOPP + LR	NED (50)
Z.G.	M	14	IA	M	M, L	MOPP	NED (90)
G.V.	F	24	IA	M	M, SC, I, IN	MOPP	AAD (6)

SC = Supraclavicular; M = mediastinum; C = cervical; A = axilla; L = lung; P = pleura; I = inguen; IN = iliac nodes; IC = infraclavicular; LR = local radiotherapy; NED = no evidence of disease; AAD = alive with active disease.

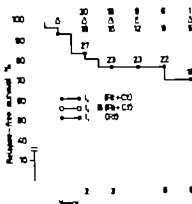


Fig. 1. Relapse-free survival of patients treated by radiotherapy and chemotherapy (Rt + Ct) and by radiotherapy alone (Rt). All patients, both I, II A and I, II B, treated by Rt + Ct are relapse-free. In contrast, 9 of 31 patients treated by Rt alone have relapsed. The difference between the curves is significant (Logrank test, $p < 0.0002$). Numbers indicate the patients relapse free at each time interval

diated lymph nodes (inguinal plus iliac nodes), 2 cases of irradiated lymph nodes, 4 cases of irradiated lymph nodes plus extranodal involvement and 1 case of extranodal relapse (lung) Mediastinum and lung were the most common sites of relapse.

Complications

Hematological toxicity was negligible and clinically uneventful in patients who received radiotherapy alone. Among the 35 patients with stage I and II A submitted to radiotherapy and MOPP 3 could not receive all the courses scheduled because of hematological toxicity in 2 cases (isolated granulocytopenia in 1 case and granulocytopenia associated to thrombocytopenia in the other). Among the 19 I II, II_E stage B patients, only 1 interrupted chemotherapy for severe neutropenia associated with infectious complications.

The frequency of herpes zoster infection was similar in each group of patients. 4 cases among those who received only radiotherapy 4 and 2 cases, respectively among I II A and I, II II_E B patients who received radiotherapy and chemotherapy

Discussion

Patients with stage I and II A, LP or NS HD treated by radiotherapy alone, have a 5-year survival of about 90% and relapse-free survival of about 70–80% [1 5 7 13] These two histological types have been usually considered to have a similar prognosis, and therefore have been treated in the same way However if radiotherapy alone often gives 100% of overall and relapse-free survival in patients with LP NS patients seldom exceed 80% of relapse-free survival [1 7 10, 13] Our previous experience confirms such data: all our patients with stage I and II A LP treated by radiotherapy alone, are alive and relapse-free while 30% of patients with stage I and II A NS, treated with radiotherapy alone, relapsed. In contrast, all patients stage I II and II_E B NS, treated by radiotherapy and MOPP are relapse-free For this reason we have submitted the patients stage I and II A NS to radiotherapy followed by only 3 courses of MOPP with the aim of reducing the relapse rate while keeping at the minimum the complications associated with added chemotherapy It is well known that 6 courses of MOPP when given after radiotherapy are myelotoxic and immunosuppressive and can significantly increase the risk of a second malignancy [13]

The results obtained in our 54 patients (35 with stage I II A NS plus 19 with stage I II II_E B), treated with radiotherapy and 3 courses of MOPP are satisfactory since

all are alive and relapse free after 20–84 months No major or life threatening complications due to either myeloid or immunologic failure occurred, nor did we observe a second malignancy Moreover the theoretic risk from the use of MOPP chemotherapy after radiotherapy is particularly evident after TNI and 6 courses of MOPP From our experience, the omission of pelvic irradiation and the use of only 3 courses of MOPP reduces significantly the risk of major complications.

At present we cannot evaluate how important relapse-free survival is in the treatment of HD but we think that the best hope for cure still depends on the adequacy of initial treatment. The ultimate goal of treatment in patients with HD should be preventing relapse without increasing morbidity or mortality From our data it appears that, at least in patients with stage I II A and B NS this goal can be reached with radiotherapy followed by only 3 courses of MOPP whereas patients with stage I A and without mediastinal involvement probably do not need additional chemotherapy

References

1 Carmel, R. J and Kaplan, H. S. Mantle irradiation in Hodgkin's disease. An analysis of technique, tumor eradication, and complications Cancer *37* 2813–2825 (1976)

2 Carbone, P P Kaplan, H S. Musshoff, K. Smithers, D W and Tubiana, M Report of the Committee on Hodgkin's disease staging classification Cancer Res. *31* 1860–1861 (1971)

3 Castellino, R. A., Glatstein, E., Turbow M M Rosenberg, S., and Kaplan, H. S Latent radiation injury of lungs or heart activated by steroid withdrawal. Ann. Intern. Med. *80* 593–599 (1974)

4 De Vita, V T., Serpick, A. A and Carbone, P P Combination chemotherapy in the treatment of advanced Hodgkin s disease. Ann. intern. Med. *73* 881–895 (1970).

5 Goodmann, R. L., Piro, A. J and Hellman, S. Can pelvic irradiation be omitted in patients with pathologic stage I A and II A Hodgkin s disease? Cancer *37* 2834–2839 (1976)

6 Johnson, R. E., Thomas, L. B Schneiderman, M Glenn, D W Faw F and Haferman, M D Preliminary experience with total nodal irradiation in Hodgkin s disease. Radiology 96 603–608 (1970)

7 Johnson, R. E. Zimbler H., Berard, C. W Herdt, J and Brereton, H. D Radiotherapy results for nodular sclerosing Hodgkin s disease after clinical staging. Cancer 39 1439–1444 (1977)

8 Kaplan, E. S. and Meier P Non-parametric estimations from incomplete observations. Am. Stat. Ass. J *53* 457–480 (1958).

9 Lukes, R. J Craver L. F Hall, T C. Rappaport, H and Ruben, T Report of the Nomenclature Committee. Cancer Res. *26*. 1311 (1966)

10 O'Connel, M. J Wiernik, P H Brace, K. C., Byhardt, R. W and Greene, W H. A combined modality approach to the treatment of Hodgkin s disease Cancer *35* 1055–1065 (1975)

11 Peto, R. Pike, M C. Breslow N E., Cox, D R Howard, S. V Mantel, N McPherson, K., Peto, J and Smith, P G Design and analysis of randomized clinical trials requiring prolonged observation of each patient. II. Analysis and examples. Br J Cancer *35* 1–39 (1977).

12 Prosnitz, L., Hellman, S. Von Eason, C., and Kligerman, M. The clinical course of Hodgkin s disease and other malignant lymphomas treated with radical radiation therapy Am. J Roentg. *105* 618–628 (1969)

13 Rosenberg, S. A. and Kaplan, H. S. The management of stages I, II and III Hodgkin's disease with combined radiotherapy and chemotherapy Cancer 35 55–63 (1975).

Received January 15 1979
Accepted May 10, 1979

Prof Sante Tura, Servizio di Ematologia, Ospedale Policlinico S. Orsola, Via Massarenti, 9 I-40138 Bologna (Italy)

Acta haemat. 62: 267-272 (1979)

Ferritin Deposits in Peripheral Blood Lymphocytes of Hodgkin's Disease Patients

Isaac Ben-Bassat, Bracha Ramot, Esther Aghai and Meir Djaldetti

Institute of Hematology, Chaim Sheba Medical Center, Tel Hashomer; Electron Microscopy Unit, Hasharon Hospital, Petah Tikva, and Sackler School of Medicine, Tel Aviv University, Tel Aviv

Key Words. Ferritin · Hodgkin's disease · Lymphocytes

Abstract. In view of the reported associations of Hodgkin's disease and ferritin, an electron microscopic study of the peripheral blood lymphocytes of these patients was done. In 5 out of the 6 patients studied intracellular ferritin deposits were seen. No such deposits were seen in the lymphocytes of healthy subjects or in those of a patient with β-thalassemia. The lymphocyte ferritin accumulation in Hodgkin's disease can arise either from increased synthesis or from phagocytosis.

Several observations reported in recent years have associated Hodgkin's disease (HD) with ferritin. Elevated serum concentrations of ferritin are consistently found in HD patients and it has been suggested that ferritin is a circulating tumor-associated antigen [2, 3, 8]. A HD-associated antigen, regularly demonstrable in high concentrations in tumor tissue, has been subsequently identified as ferritin [6]. Recently splenic tumor cells [14] and peripheral blood lymphocytes [15] have been shown to have an increased ferritin synthesis.

We have shown that in HD there is a subpopulation of peripheral blood T lymphocytes that does not form E rosettes but can be unblocked by levamisole and that ferritin is shed from the surface of the treated lymphocytes [1, 13]. In view of all these observations we undertook an electron microscopic study of peripheral blood lymphocytes of HD patients.

Materials and Methods

Peripheral blood mononuclear cells were separated on Ficoll-Hypaque gradient [4] and fixed in cold 1% glutaraldehyde in phosphate buffer pH 7.4. The cells were postfixed in osmium tetroxide, dehydrated in graded alcohols and embedded in Epon 812. Thin sections were cut with an LKB ultratome III and examined with a Philips 300 electron microscope.

Patients

6 HD patients were studied: 1 was newly diagnosed and studied before treatment was started

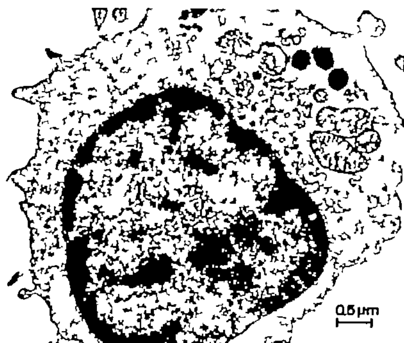


Fig. 1. Peripheral blood lymphocyte containing a few iron deposits. × 20,000.

Fig. 2. A lymphocyte containing iron deposits with high (curved rrow) nd lower (straight arrows) electron density × 62,200.

and 5 were in remission, 1–4 years after the completion of therapy. At diagnosis 1 patient was in stage I, 4 in stage II and 1 in stage III. As controls the lymphocytes of 50 healthy subjects and patient with homozygous β-thalassemia with iron overload were studied.

Results

No ferritin deposits were seen in the lymphocytes of 50 healthy subjects or in the thalassemic patient. In 5 out of the 6

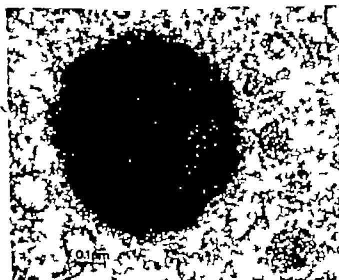


Fig. 3. Higher magnification of ferritin cluster without limiting membrane. 155,400



Fig. 4. Ferritin cluster with surrounding membrane. 227,125

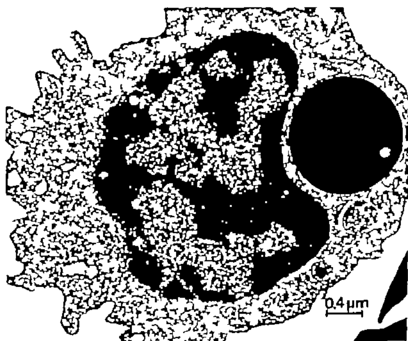


Fig. 5. A lymphocyte with a phagocytized body. × 21,800.

HD patients intracellular ferritin deposits were seen in the peripheral lymphocytes. Figure 1 represents a lymphocyte containing a few iron deposits. The ultrastructure of the cell appears quite normal. In some cells (fig. 2) two types of deposits were noted: one with marked electron density, the second with more pronounced granular appearance. On higher magnification, the iron deposits represented ferritin clusters without (fig. 3) or with (fig. 4) a surrounding membrane. In 1 patient some of the lymphocytes also contained phagocytized material (fig. 5).

Discussion

In previous studies we have demonstrated that the immunomodulating drug levamisole raises the E rosette-forming cell number of HD patients [13]. By iodination of the lymphocyte membrane followed by levamisole treatment it has been shown that ferritin is one of the proteins shed from the lymphocyte membrane [12]. It was therefore of interest to see whether ferritin could be detected on the surface of the lymphocyte membrane of these patients. Although membrane bound ferritin was not detected, quite remarkable intracellular accumulation of ferritin was observed in the lymphocytes of 5 out of 6 HD patients. No ferritin deposits were seen in the peripheral blood lymphocytes of normal subjects or in the lymphocytes of a homozygous β-thalassemia patient with systemic iron overload. As lymphocytes are not reticuloendothelial cells, this finding is not a nonspecific iron accumulation as seen in chronic disorders. *Dumont et al.* [5] described marked deposition of iron in involved and uninvolved HD lymph nodes and particularly in those with nodular sclerosis type. The deposits seen by light microscopy were cellular, stromal and in the fibrous tissue. *Sarcione et al.* [15] have shown that HD peripheral blood lymphocytes synthesize ferritin 4.2 times faster

and release it 2.4 times faster than normal lymphocytes. No relationship was observed between relative rates of lymphocyte ferritin synthesis and sex, age or pathologic stage of the disease. These data suggest that the iron deposits seen by electron microscopy could be the result of increased synthesis. However in view of the phagocytic activity of some of these lymphocytes (fig. 5), pinocytosis or phagocytosis of ferritin by the lymphocytes cannot be excluded. Phagocytic activity by normal peripheral blood lymphocytes [9] acute leukemia lymphoblasts [7] and hairy-cell leukemia cells [16] has been previously described but has not, to the best of our knowledge, been reported in HD lymphocytes.

Increased ferritin levels are present in several human tumor cells like hepatoma [1] carcinoma of the breast and pancreas [10] The interesting point in our observation is that the iron deposits are found in cells not considered to be malignant and their presence is unrelated to the disease activity

The significance of these morphologic findings to the immunologic abberations in HD is unknown and should be the subject for further study It should be noted, however that ferritin can significantly suppress the mitogen-induced blastic transformation of normal lymphocytes [11]

References

1 Alpert, E., Coston, R. L., and Drysdale, J. W Carcino-foetal human liver ferritin. Nature, Lond 4 194–195 (1973)

2 Arnoult, C W Ferritin in body fluids. J Lab. clin. Med 71 517–522 (1968)

3 Bieber C. P and Bieber M. M Detection of ferritin as circulating tumor-associated antigen in Hodgkin's disease. Natn. Cancer Inst. Monogr 36 145–157 (1973).

4 Boyum, A Isolation of mononuclear cells and granulocytes from human blood. Scand. J clin. Lab. Invest 21 suppl. 97 pp. 77–89 (1968).

5 Dumont, A. E., Ford, R. J and Becker F F Siderosis of lymph nodes in patients with Hodgkin disease. Cancer 38 1247–1252 (1976).

6 Eshhar Z., Order S E., and Katz, D H. Ferritin, Hodgkin disease associated antigen. Proc natn. Acad. Sci. USA 71 3956–3960 (1974)

7 Foadi, M. D Slater A. M., and Pegrum, G. D Erythrophagocytosis by acute lymphoblastic leukaemic cells. Scand. J Haematol. 20 85–88 (1978).

8 Jones, P A. E., Miller F M Worwood, M., and Jacobs, A. Ferritinaemia in leukaemia and Hodgkin disease Br J Cancer 27 212 217 (1973)

9 Kozarewski, B. J Emerick, C W and Dienn, D R. Studies of phagocytic activity of lymphocytes III. Phagocytosis of intravenous India ink in human subjects Blood 12 559–566 (1957).

10 Marcus, D M. and Zinberg, N Isolation of ferritin from human mammary and pancreatic carcinomas by means of antibody immunoadsorbents Archs Biochem. Biophys 162 493–501 (1974).

11 Matzner Y Hershko, C., Polliack, A., Konijn, A M and Izak, G Suppressive effect of ferritin on in vitro lymphocyte function. Br J Haemat. (in press, 1979).

12 Moroz, C., Lahat, N Biniaminov M and Ramot, B., Ferritin on the surface of lymphocytes in Hodgkin disease patients. A possible blocking substance removed by levamisole Clin exp. Immunol. 29 30–36 (1977).

13 Ramot, B Biniaminov M., Shoham, C., and Rosenthal, E Effect of levamisole on E-rosette forming cells in vivo and in vitro in Hodgkin disease. New Engl. J Med 294 809–811 (1976).

14 Sarcione, E. J Stutzman, L., and Mittelman, A Ferritin synthesis by splenic tumor tissue of Hodgkin disease. Experientia 31 1334–1335 (1975)

15 Sarcione, E. J Smalley J R., Lema, M. J and Stutzman, L. Increased ferritin synthesis

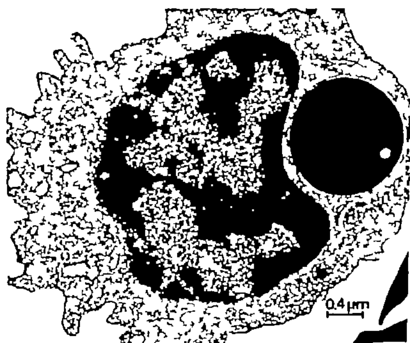


Fig. 5 A lymphocyte with a phagocytized body × 21,800.

HD patients, intracellular ferritin deposits were seen in the peripheral lymphocytes. Figure 1 represents a lymphocyte containing a few iron deposits. The ultrastructure of the cell appears quite normal. In some cells (fig. 2) two types of deposits were noted: one with marked electron density, the second with more pronounced granular appearance. On higher magnification, the iron deposits represented ferritin clusters without (fig. 3) or with (fig. 4) a surrounding membrane. In 1 patient some of the lymphocytes also contained phagocytized material (fig. 5).

Discussion

In previous studies we have demonstrated that the immunomodulating drug levamisole raises the E rosette-forming cell number of HD patients [13]. By iodination of the lymphocyte membrane followed by levamisole treatment it has been shown that ferritin is one of the proteins shed from the lymphocyte membrane [12]. It was therefore of interest to see whether ferritin could be detected on the surface of the lymphocyte membrane of these patients. Although membrane-bound ferritin was not detected, quite remarkable intracellular accumulation of ferritin was observed in the lymphocytes of 5 out of 6 HD patients. No ferritin deposits were seen in the peripheral blood lymphocytes of normal subjects or in the lymphocytes of a homozygous β-thalassemia patient with systemic iron overload. As lymphocytes are not reticuloendothelial cells, this finding is not a nonspecific iron accumulation as seen in chronic disorders. *Dumont et al.* [5] described marked deposition of iron in involved and uninvolved HD lymph nodes and particularly in those with nodular sclerosis type. The deposits seen by light microscopy were cellular, stromal and in the fibrous tissue. *Sarcione et al.* [15] have shown that HD peripheral blood lymphocytes synthesize ferritin 4.2 times faster

Acta haemat. 62, 273-277 (1979)

Variation of Serum Ferritin in Low Birth Weight Infants with Maternal Ferritin, Birth Weight and Gestational Age

Lennart Jansson, Lars Holmberg and Rolf Ekman

Department of Paediatrics, University of Lund, Department of Clinical Chemistry University Hospital, Lund, and Malmo General Hospital, Malmo

Key Words. Iron stores Low birth weight Serum ferritin

Abstract. Serum ferritin measured at birth in 69 low birth weight infants proved to vary with gestational age as well as with weight. The increase with gestational age was even more striking when the infants small for gestational age were excluded. The relation between maternal and infant serum ferritin concentration was investigated for 2 groups of infants and their mothers (preterm and term infants, respectively) Neither in preterm nor in term infants was the serum ferritin found to vary with that in the respective mothers.

The serum ferritin (s-ferritin) assay has proved to be a simple and quantitative method for measuring iron stores in normal development as well as in iron deficiency and iron overload [3 6, 13 15] Until now this technique has been applied in only few investigations of newborns and never in preterm infants. In the present study we investigated whether the level of s-ferritin in low birth weight (LBW) infants of various gestational ages (GA) was in keeping with known data on iron stores in such infants. We also studied the maternal iron stores for any covariation with those in LBW infants, measured as s-ferritin. For comparison, maternal and cord blood s-ferritin from normal term infants were also studied.

Patients and Methods

The clinical material consisted of 69 LBW infants and 20 normal term infants. Of the LBW infants, 63 were born before term and 6 at term. The distribution of the 63 preterm infants according to GA was as follows: < 32 weeks: 9 32 33 weeks: 12, 34-35 weeks 4 36-37 weeks: 18. Eight of the preterm infants were classified as small for gestational age (SGA) and 55 were appropriate for gestational age (AGA). The distribution of the 69 LBW infants according to birth weight (BW) was as follows: < 1,500 g: 12, 1,500-1,999 g: 19 2,000-2,499 g: 38 All the infants were born in Malmö General Hospital the LBW infants between December 1975 and January 1977 the normal term infants in November 1977 The clamping of the umbilical cord was done not earlier than 1 min and not later than 5 min after delivery Of the LBW infants, 6 developed signs of

and release by Hodgkin's disease peripheral blood lymphocytes. Int. J Cancer *20* 339–346 (1977)
16 Utsinger P D., Jount, W J., Fuller C. R., Logue, M J and Orringer E. P., Hairy cell leukemia. B-lymphocyte and phagocytic properties. Blood *49* 19–27 (1977).

Received March 13, 1979
Accepted. March 23, 1979

Isaac Ben-Bassat, MD Institute of Hematology
Chaim Sheba Medical Center
Tel Hashomer (Israel)

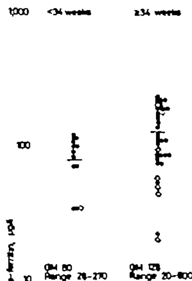


Fig. 2. s-ferritin in preterm infants versus gestational age. ● = AGA infants; ◊ = SGA infants. Geometrical means and ranges are shown in the figure. p values when comparing the two groups, SGA infants included p < 0.01. SGA infants excluded p < 0.0025.

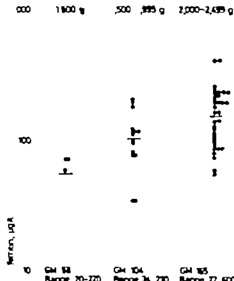


Fig. 3. s-ferritin in LBW infants versus BW. The geometrical means and ranges are shown in the figure. Infants < 1,500 g vs. infants 1,500–1,999 g: p < 0.01; infants 1,500–1,999 g vs. infants 2,000–2,499 g: p < 0.025.

SGA infants were excluded the difference between the groups was even more striking (p < 0.0025).

The relationship between s-ferritin at 24–48 h and BW is shown in figure 3. Analyses of the values showed that s-ferritin increased with BW: infants < 1,500 g vs. infants 1,500–1,999 g: p < 0.01; infants 1,500–1,999 g vs. infants 2,000–2,499 g: p < 0.025.

Discussion

The maternal s-ferritin values found here are consistent with those on record [5, 7]. The effect of maternal iron deficiency on the iron stores in the fetus is still debatable. *Rios et al.* [11] and *Hussain et al.* [5] found no correlation between maternal and infant s-ferritin whether the mothers had signs of depleted iron stores or not. Recently, however, *Fenton et al.* [4] found cord s-ferritin to be lower in infants born of mothers with low s-ferritin (< 12 µg/l). We found no correlation between maternal and infant s-ferritin levels whether the infants were born at or before term. However, our series, like that reported by *Rios et al.* [11], included only a few mothers of term infants and none of preterm infants with s-ferritin values indicating depletion of iron stores. This may explain the differences between the results of the investigations.

respiratory distress and 10 were given phototherapy because of hyperbilirubinaemia. None of the infants showed any sign of hepatic or renal disease.

Also the mothers of 23 of the preterm, and of all the 20 normal term, infants were investigated. All the mothers had received iron supplementation from early pregnancy (Duroferon vitamin® AB Hässle, Sweden, supplying 200 mg ferrous sulphate/day). None of the mothers showed any clinical sign of malignant, hepatic or renal disease.

The GA of each infant was assessed from maternal data and external characteristics [14] and from a neurological evaluation [1].

In the LBW group s-ferritin was determined in cord blood (23 infants) and in blood obtained by heal puncture at 24–48 h of age (all 69 infants). In the mothers, the determination was made in blood obtained by venipuncture immediately after delivery. In the normal term infants cord blood was used for ethical and practical reasons. Maternal blood was drawn as described above. Sera was stored at −70 C until analysed. S-ferritin was determined according to the method of *Miles et al.* [9] with reagents from Ramco, Laboratories Inc., Houston, Tex. Because of the skewed normal distribution of s-ferritin [3, 6, 8, 13] the statistical analyses were performed after logarithmic transformation. The means are therefore expressed as geometrical means (GM). The significances of differences between the groups were calculated with Student's t test.

Results

S-ferritin was determined in 23 preterm infants with a mean GA of 35 weeks (range 32–37 weeks) both in cord blood and at 24–48 h of age. All the mothers of these infants had normal s-ferritin values (GM 49, range 15–160 μg/l). The corresponding s-ferritin values of their infants rose from a GM of 54 (range 10–192 μg/l) in cord blood to a GM of 121 (range 20–340 μg/l) at 24–48 h of age. There was no correlation between maternal and infant s-ferritin either in cord blood ($r = 0.25$) or at 24–48 h of age ($r = 0.01$).

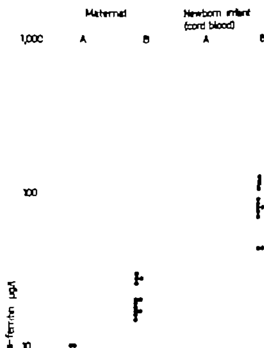


Fig. 1. s-ferritin in two groups of normal mothers at the time of delivery and the corresponding values for their newborn term infants. A = Maternal s-ferritin < 12 μg/l. B = maternal s-ferritin > 12 μg/l.

Of the mothers of 20 normal infants, the s-ferritin in 4 was < 12 μg/l. Such a low level has previously been found to indicate depletion of the iron stores [6]. The infants of these mothers were divided into two groups: (A) maternal s-ferritin < 12 μg/l; (B) maternal s-ferritin > 12 μg/l. The s-ferritin level in infants did not vary with that in the mothers (fig. 1).

The relation between s-ferritin at 24–48 h and GA in 63 preterm infants is shown in figure 2. The infants were divided into two groups: (A) GA < 34 weeks; (B) GA ≥ 34 weeks. These groups differed from each other in s-ferritin ($p < 0.01$). Of the 8 SGA infants, all but 2 had s-ferritin values below the GM for their group. When the

14 Usher R., McLean, F and Scott, K.. Judgement of foetal age. II. Clinical significance of gestational age and objective method for its assessment. Pediat. Clins N Am. *13* 835–848 (1966).
15 Walters, G O Miller F M, and Worwood, M. Serum ferritin concentration and iron stores in normal subjects. J clin Path *26* 770–772 (1973).
16 Widdowson, E. M. and Spray C. M Chemical development in utero. Archs Dis. Child. *26*. 205–214 (1951).

Received. March 15 1979
Accepted: June 20, 1979

Dr L. Jansson, Department of Paediatrics,
Malmo General Hospital,
S-21401 Malmo (Sweden)

The iron content of the fetus as well as of the newborn infant has received wide attention *Widdowsson and Spray* [16] found a close correlation between iron content and body weight, a finding corroborated by *Osgood* [10] and *Chang* [2] *Seip and Halvorsen* [12] found that bone marrow from infants with a BW below 1 400 g contained less stainable iron than that from larger infants In the present study iron stores were assessed from the s-ferritin concentration which has proved to be a good indicator of iron stores in adults children and infants under basal conditions [3 6 13 15]

After birth there are rapid changes in blood volume and erythropoiesis These changes are also reflected in s-ferritin. In fullterm infants *Rios et al* [11] found a rise in s-ferritin during the first day of life, but thereafter the level was constant at least for 1 week. The 24–48 h s-ferritin values, therefore, probably are more representative of the iron stores at birth than cord blood values We found the same rise in s-ferritin during the first 24 h of life in preterm infants as *Rios et al* [11] did in fullterm infants Thus we have chosen the 24-hour values for comparison between the different GA and BW group We found a clear correlation between s-ferritin and BW as well as between s-ferritin and GA. However the range in all groups was wide and the values showed the same skewed distribution as that found in older children and adults. Hence, it appears that it is not warranted to assess the iron stores in a given infant from the s-ferritin alone For ethical reasons, however it was not possible in the present investigation to study the s-ferritin levels for any correlation with other indicators of iron stores, such as the amount of stainable bone marrow iron

References

1 Amiel-Tison, C. Neurologic evaluation of the maturity of newborn infants. Archs Dis. Child *43* 89–93 (1968).

2 Chang, L. L. Storage iron in foetal livers. Acta paediat. scand *62*. 173–175 (1973).

3 Cook, J D Lipschitz, D A. Miles, L. E. M. and Finch, C. A. Serum ferritin as a measure of iron stores in normal subjects. Am. J clin Nutr *27* 681–687 (1974)

4 Fenton, V Cavill, I, and Fisher J Iron stores in pregnancy Br J Haemat. *37* 145–148 (1977).

5 Hussain, M. A. Gaafar T H., Laulicht, M, and Hoffbrand, A. V Relation of maternal and cord blood serum ferritin. Archs Dis Child. *52* 782–784 (1977).

6 Jacobs, A., Miller F., Worwood, M., Beamish, M R., and Wardrop, M R.. Ferritin in the serum of normal subjects and patients with iron deficiency and iron overload. Br med. J *iv* 206–208 (1972)

7 Kelly A. M MacDonald, D J and McNay M B Ferritin as an assessment of iron stores in normal pregnancy Br J Obstet. Gynaecol. *84* 434–438 (1977).

8 Lipschitz, D A Cook, J D and Finch, C. A. A clinical evaluation of serum ferritin as an index of iron stores. New Engl. J Med *290* 1213–1216 (1974)

9 Miles, L. E. M Lipschitz, D A., Bieber C. P and Cook, J D Measurement of serum ferritin by a 2-site immunoradiometric assay Analyt. Biochem. *61* 209–224 (1974).

10 Osgood, E. E. Development and growth of haematopoietic tissues. Pediatrics *15* 733–751 (1955).

11 Rios, E. Lipschitz, D A., Cook, J D and Smith, N J Relationship of maternal and infant iron stores by determination of plasma ferritin. Pediatrics *55* 694–699 (1975).

12 Seip, M and Halvorsen, S.. Erythrocyte production and iron stores in premature infants during the first months of life. Acta paediat. scand. *45* 600–617 (1956).

13 Siimes, M A. Addiego, J E., and Dallman, P R.. Ferritin in serum diagnosis of iron deficiency and iron overload in infants and children. Blood *43* 581–590 (1974).

Serum and erythrocytes were prepared and extracted as described previously [8] Preparation and extraction of organs were performed as reported in detail in previous paper [7] except that the organs were chopped and lyophilized before lipid extraction. The total lipids of adipose tissue were fractionated on silicic acid column [9], yielding polar lipid and neutral lipid fraction

Total lipids (polar lipids from adipose tissue, respectively) and nonlipid fractions of serum, erythrocytes and organs wer tested on porcine A blood group activity by their ability to inhibit the agglutination of A pig erythrocytes (test erythrocytes). The sample was emulsified with isotonic saline (yielding suspension of about 1 mg/ml) using glass homogenizer Dilution series of the emulsion up to 1.256 and controls (without anti-A) served for the hemagglutination-inhibition tests All tests were performed at least twice

Results and Discussion

Erythrocytes of 22 pigs were tested on A blood group activity About 3/4 of them were A-positive. 3 A positive pigs (No.I–III) were selected whose A activity was found in the total lipid fraction of erythrocytes. They carried the A activity also in the total lipid fraction of serum They differed from one another in that No I was A-active in the erythrocyte nonlipid fraction, but not in the serum nonlipid fraction No II was A-negative in the erythrocyte nonlipid fraction as well as in the serum nonlipid fraction No III was A-negative in the erythrocyte nonlipid fraction, but A-positive in the serum non-lipid fraction

Some tissues (myocardium, skeletal muscle brain adipose tissue) were found to be A-negative in all animals (table I). This is consistent with the way of distribution of J in J-positive cattle [7] Among the nonlipid fractions of parenchymatous organs, A activity was only detected in kidney of pig No. III Some A-positive animals carry the lipidic A substance in their parenchymatous organs (spleen, liver kidney), others not. It has been shown [2] that A negative pig erythrocytes can be converted to A-positive by incubating (5 h, 37 C) the cells in A-positive serum. It has therefore been concluded [2] that the soluble A substance of serum is the gene product which is secondarily taken up the red cell By the mode of distribution of A activity on lipids and nonlipids of serum and erythrocytes (table I), we conclude that the lipidic A substance of serum or part of it (containing the A determinant) is likely to be transferred from the serum to the erythrocyte membrane. The distribution of A activity in pig No III is consistent with the view that also a transfer from a serum lipid to a cell membrane protein may occur On the other hand, the A distribution in pig No I shows that also a transfer of A determinant from a lipid-free serum protein to a cellular lipid might occur as it was reported in the case of the bovine J determinant [6, 10]

The results indicating the occurrence of A activity in several parenchymatous organs raise the question for the origin of the A substance It may be speculated that the A substance of spleen lipids of pig No III may be derived from the membranes of old erythrocytes which have been trapped by the spleen and may resist their metabolic breakdown for a longer period. However the absence of A activity in the spleen lipids of pigs No. I and II remains then obscure. The A activity of organs may be due to one of the following reasons: the A substance may either be synthesized by those organs, or it may be absorbed by the tissue cells from the blood plasma in the same way as it

Acta haemat. 62, 278–281 (1979)

Distribution of the A Blood Group Activity on Lipid and Nonlipid Fractions of Pig Organs[1]

F. Krötlinger, J.-N. Meyer and O. W. Thiele

Physiologisch-Chemisches Institut and Institut für Tierzucht und Haustiergenetik, *University of Göttingen, Göttingen*

Key Words. Blood group · Lipids · Pig organs

Abstract. *Lipids and protein-containing nonlipids of pig erythrocytes, serum and several* organs were tested for A blood group activity. Both lipids and nonlipids of brain, myocardium, skeletal muscle and adipose tissue show no A activity. The way of distribution of A activity on lipids and nonlipids of other tissues and serum differs among individual pigs. With respect to the acquisition of A activity of erythrocytes in a postnatal period it seems likely that the lipidic A substance (or part of it containing the A determinant) is *transferred* from plasma to the erythrocyte membrane.

Introduction

There are blood group-active substances in various mammalian species that are primarily dissolved in the blood *plasma* and secondarily transferred to the erythrocyte membrane [1]. The A blood group antigen of pig [2] and the J blood group antigen of cattle [3] are examples of those kinds of antigens. It has been shown in previous papers that the J activity of cattle [4] as well as the A activity of pig [5] can be found in both a lipid and a nonlipid fraction of serum. Furthermore, it has been demonstrated [6–10] that the determinant of the nonlipid J substance of bovine serum is transferred to a lipidic receptor of the erythrocyte membrane. We have also presented a study [7] on the occurrence of lipidic and nonlipidic J antigens in various bovine organs. This paper reports on an extension of these studies to organs of pigs with respect to the lipidic and nonlipidic A substance. The results could be useful for future studies concerning the origin of the A determinant and its transfer to erythrocytes or tissue cells.

Materials and Methods

Blood and organs were taken from pigs (German Landrace) at the local slaughterhouse. To differ between A-positive and A-negative pigs, erythrocyte suspensions were tested with normal anti-A serum.

1 Supported by Forschungsmittel des Landes Niedersachsen.

Serum and erythrocytes were prepared and extracted as described previously [8]. Preparation and extraction of organs were performed as reported in detail in previous paper [7] except that the organs were chopped and lyophilized before lipid extraction. The total lipids of adipose tissue were fractionated on silicic acid column [9], yielding polar lipid and neutral lipid fraction.

Total lipids (polar lipids from adipose tissue, respectively) and nonlipid fractions of serum, erythrocytes and organs were tested on porcine A blood group activity by their ability to inhibit the agglutination of A pig erythrocytes ('test erythrocytes'). The sample was emulsified with isotonic saline (yielding suspension of about 1 mg/ml) using glass homogenizer. Dilution series of this emulsion up to 1:256 and controls (without anti-A) served for the hemagglutination-inhibition tests. All tests were performed at least twice.

Results and Discussion

Erythrocytes of 22 pigs were tested on A blood group activity. About 3/4 of them were A positive. 3 A positive pigs (No I–III) were selected whose A activity was found in the total lipid fraction of erythrocytes. They carried the A activity also in the total lipid fraction of serum. They differed from one another in that No. I was A active in the erythrocyte nonlipid fraction, but not in the serum nonlipid fraction. No. II was A-negative in the erythrocyte nonlipid fraction as well as in the serum nonlipid fraction, No. III was A-negative in the erythrocyte nonlipid fraction, but A-positive in the serum non-lipid fraction.

Some tissues (myocardium, skeletal muscle, brain, adipose tissue) were found to be A-negative in all animals (table I). This is consistent with the way of distribution of J in J-positive cattle [7]. Among the nonlipid fractions of parenchymatous organs, A activity was only detected in kidney of pig No. III. Some A-positive animals carry the lipidic A substance in their parenchymatous organs (spleen, liver kidney), others not. It has been shown [2] that A negative pig erythrocytes can be converted to A-positive by incubating (5 h, 37 C) the cells in A-positive serum. It has therefore been concluded [2] that the soluble A substance of serum is the gene product which is secondarily taken up the red cell. By the mode of distribution of A activity on lipids and nonlipids of serum and erythrocytes (table I), we conclude that the lipidic A substance of serum or part of it (containing the A determinant) is likely to be transferred from the serum to the erythrocyte membrane. The distribution of A activity in pig No. III is consistent with the view that also a transfer from a serum lipid to a cell membrane protein may occur. On the other hand, the A distribution in pig No. I shows that also a transfer of A determinant from a lipid-free serum protein to a cellular lipid might occur as it was reported in the case of the bovine J determinant [6, 10].

The results indicating the occurrence of A activity in several parenchymatous organs raise the question for the origin of the A substance. It may be speculated that the A substance of spleen lipids of pig No. III may be derived from the membranes of old erythrocytes which have been trapped by the spleen and may resist their metabolic breakdown for a longer period. However, the absence of A activity in the spleen lipids of pigs No. I and II remains then obscure. The A activity of organs may be due to one of the following reasons: the A substance may either be synthesized by those organs, or it may be absorbed by the tissue cells from the blood plasma in the same way as it

Acta haemat. 62 278–281 (1979)

Distribution of the A Blood Group Activity on Lipid and Nonlipid Fractions of Pig Organs[1]

F. Krötlinger, J. N. Meyer and O. W. Thiele

Physiologisch-Chemisches Institut and Institut für Tierzucht und Haustiergenetik, University of Göttingen, Göttingen

Key Words. Blood group · Lipids · Pig organs

Abstract. Lipids and protein-containing nonlipids of pig erythrocytes, serum and several organs were tested for A blood group activity. Both lipids and nonlipids of brain, myocardium, skeletal muscle and adipose tissue show no A activity. The way of distribution of A activity on lipids and nonlipids of other tissues and serum differs among individual pigs. With respect to the acquisition of A activity of erythrocytes in a postnatal period, it seems likely that the lipidic A substance (or part of it containing the A determinant) is transferred from plasma to the erythrocyte membrane.

Introduction

There are blood group-active substances in various mammalian species that are primarily dissolved in the blood plasma and secondarily transferred to the erythrocyte membrane [1]. The A blood group antigen of pig [2] and the J blood group antigen of cattle [3] are examples of those kinds of antigens. It has been shown in previous papers that the J activity of cattle [4] as well as the A activity of pig [5] can be found in both a lipid and a nonlipid fraction of serum. Furthermore, it has been demonstrated [6–10] that the determinant of the nonlipid J substance of bovine serum is transferred to a lipidic receptor of the erythrocyte membrane. We have also presented a study [7] on the occurrence of lipidic and nonlipidic J antigens in various bovine organs. This paper reports on an extension of these studies to organs of pigs with respect to the lipidic and nonlipidic A substance. The results could be useful for future studies concerning the origin of the A determinant and its transfer to erythrocytes or tissue cells.

[1] Supported by Forschungsmittel des Landes Niedersachsen.

Materials and Methods

Blood and organs were taken from pigs (German Landrace) at the local slaughterhouse. To differ between A-positive and A-negative pigs, erythrocyte suspensions were tested with normal anti-A serum.

Agglutination in the presence of

lipid-free residues from µg lyophilized organs

660	330	165	80	40	20
−	+	+++	+++	+++	+++
+++	+++	+++	+++	+++	+++
+++	+++	+++	+++	+++	+++
+++	+++	+++	+++	+++	+++
+++	+++	+++	+++	+++	+++
−	+	+	+++	+++	+++
+++	+++	+++	+++	+++	+++
+++	+++	+++	+++	+++	+++
+++	+++	+++	+++	+++	+++
+++	+++	+++	+++	+++	+++
+++	+++	+++	+++	+++	+++
+++	+++	+++	+++	+++	+++
+++	+++	+++	+++	+++	+++
+++	+++	+++	+++	+++	+++
−	+	+	+++	+++	+++
+++	+++	+++	+++	+++	+++
+++	+++	+++	+++	+++	+++
++	++	+++	+++	+++	+++
+++	+++	+++	+++	+++	+++

the studies in animals with J substance in serum and erythrocytes and animals with J only in serum. Eur J Biochem. 22 396–399 (1971).

5 Thiele, O W and Homf J The chemical nature of the pig blood-group substances dissolved in the serum. Experientia 27 447 (1971).

6 Thiele, O W Krotlinger F and Ohl, C. Transfer of blood-group determinants from plasma onto erythrocytes. Naturwissenschaften 62. 586 (1975).

7 Thiele, O W Froneberg, B., and Koch, J Occurrence of J blood-group activities in lipid and nonlipid fractions of organs and body fluids of cattle. Anim. Blood Grps biochem. Genet. 5 215–224 (1974).

8 Schroffel, J Thiele, O W and Koch J Studies on the attachment of the bovine J blood-group substance at the erythrocyt membrane. Eur J Biochem. 22. 294–300 (1971).

9 Borgström, B. Investigation on lipid separation methods Acta physiol. scand. 25 101–110 (1952)

10 Krotlinger F Thiele, O W and Ohl, C. The transfer of J blood-group activity from J containing bovine serum to J negative erythrocytes. Eur J Biochem. 67 495–502 (1976).

Received March 6, 1979
Accepted May 10, 1979

Prof Dr O. W Thiele,
Physiologisch-Chemisches Institut der
Georg August-Universität, Humboldtallee 7
D-34 Göttingen (FRG)

Table I. Inhibition of agglutination of A+ test erythrocytes by total lipids or lipid-free residues from various pig organs animals, which were A-negative in erythrocytes, were also A-negative in all organs mentioned

Organ	Animal No.	Agglutination in the presence of total lipids from µg lyophilized organs					
		660	330	165	80	40	20
Erythrocytes	I	−	+	+	+	++	+++
	II	−	+	+++	+++	+++	+++
	III	−	+	++	+++	+++	+++
Serum	I	−	+	+++	+++	+++	+++
	II	−	−	+++	+++	+++	+++
	III	−	−	+	+++	+++	+++
Spleen	I	+++	+++	+++	+++	+++	+++
	II	+++	+++	+++	+++	+++	+++
	III	−	−	++	+++	+++	+++
Liver	I	+++	+++	+++	+++	+++	+++
	II	−	−	+	++	+++	+++
	III	−	−	−	++	+++	+++
Kidney	I	+++	+++	+++	+++	+++	+++
	II	−	−	++	+++	+++	+++
	III	−	−	−	++	+++	+++
Lymph nodules	I	+	++	+++	+++	+++	+++
	II	−	−	+++	+++	+++	+++
	III	−	−	−	−	++	+++
Myocardium, Skeletal muscle, Brain, Adipose tissue (perirenal subcutaneous)	I III	+++	+++	+++	+++	+++	+++

− = No agglutination in the pig A system (complete inhibition) + + + = complete agglutination in the pig A system (no inhibition) + + + = intermediate degrees of agglutination (partial inhibition).

[1] Polar lipids instead of total lipids.

is acquired by the erythrocytes. In this case, the absorbing tissue cells are suggested to hold special receptor sites at their membrane surface while tissues without A activity (e.g. liver and kidney of pig No I or brain and muscle tissue of all animals) are probably lacking such receptor sites. The above results stimulate detailed studies on the transfer mechanism of the A determinant from plasma to the cell membrane.

References

1 Thiele, O W Lösliche Blutgruppenantigene von Glykolipidcharakter Blut *24* 314–320 (1972).

Andresen, E. Blood groups in pigs. Ann. N. Y Acad. Sci. *97* 205–225 (1962).

3 Stormont, C. Acquisition of the J substance by the bovine erythrocyte. Proc. natn. Acad. Sci. USA *35* 232–237 (1949)

4 Schröffel, J., Radal, A., Thiele O W and Koch, J The J blood-group substance of cat

Agglutination in the presence of

lipid-free residues from µg lyophilized organs

660	330	165	80	40	20
−	+	+++	+++	+++	+++
+++	+++	+++	+++	+++	+++
+++	+++	+++	+++	+++	+++
+++	+++	+++	+++	+++	+++
+++	+++	+++	+++	+++	+++
−	+	+	+++	+++	+++
+++	+++	+++	+++	+++	+++
+++	+++	+++	+++	+++	+++
+++	+++	+++	+++	+++	+++
+++	+++	+++	+++	+++	+++
+++	+++	+++	+++	+++	+++
+++	+++	+++	+++	+++	+++
+++	+++	+++	+++	+++	+++
+++	+++	+++	+++	+++	+++
−	+	+	+++	+++	+++
+++	+++	+++	+++	+++	+++
+++	+++	+++	+++	+++	+++
++	++	+++	+++	+++	+++
+++	+++	+++	+++	+++	+++

ties: studies in animals with J substance in serum and erythrocytes and animals with J only in serum. Eur J Biochem. 22. 396–399 (1971).

5 Thiele, O W and Holný J The chemical nature of the pig blood-group substances dissolved in the serum. Experientia 27 447 (1971)

6 Thiele, O W Kröllinger F and Ohl, C. Transfer of blood-group determinants from plasma onto erythrocytes. Naturwissenschaften 62 586 (1975).

7 Thiele, O W Froneberg, B., and Koch, J Occurrence of J blood-group activities in lipid and nonlipid fractions of organs and body fluids of cattle. Anim. Blood Grps biochem Genet 5 215–224 (1974).

8 Schroffl I, J Thiele, O W and Koch J Studies on the attachment of the bovine J blood-group substance at the erythrocyte membrane. Eur J Biochem. 22. 294–300 (1971).

9 Borgstrom, B Investigation on lipid separation methods. Acta physiol. scand 25 101 110 (1952)

10 Kröllinger F Thiele, O W and Ohl, C. The transfer of J blood-group activity from J containing bovine serum to J negative erythrocytes. Eur J Biochem 67 495–502 (1976).

Received March 6, 1979
Accepted May 10 1979

Prof Dr O W Thiele,
Physiologisch-Chemisches Institut der
Georg-August-Universität, Humboldtallee 7
D-34 Göttingen (FRG)

Table I Inhibition of agglutination of A test erythrocytes by total lipids or lipid-free residues from various pig organs animals, which were A-negative in erythrocytes, were also A-negative in all organs mentioned

Organ	Animal No.	Agglutination in the presence of total lipids from µg lyophilized organs					
		660	330	165	80	40	20
Erythrocytes	I	−	+	+	+	++	+++
	II	−	+	+++	+++	+++	+++
	III	−	+	++	+++	+++	+++
Serum	I	−	+	+++	+++	+++	+++
	II	−	−	+++	+++	+++	+++
	III	−	−	+	+++	+++	+++
Spleen	I	+++	+++	+++	+++	+++	+++
	II	+++	+++	+++	+++	+++	+++
	III	−	−	++	+++	+++	+++
Liver	I	+++	+++	+++	+++	+++	+++
	II	−	−	+	++	+++	+++
	III	−	−	−	++	+++	+++
Kidney	I	+++	+++	+++	+++	+++	+++
	II	−	−	++	+++	+++	+++
	III	−	−	−	++	+++	+++
Lymph nodules	I	+	++	+++	+++	+++	+++
	II	−	−	+++	+++	+++	+++
	III	−	−	−	−	++	+++
Myocardium Skeletal muscle, Brain, Adipose tissue (perirenal[1] subcutaneous[1])	I III	+++	+++	+++	+++	+++	+++

− = No agglutination in the pig A system (complete inhibition) +++ = complete agglutination in the pig A system (no inhibition) + ++ = intermediate degrees of agglutination (partial inhibition).
Polar lipids instead of total lipids.

is acquired by the erythrocytes. In this case, the absorbing tissue cells are suggested to hold special receptor sites at their membrane surface while tissues without A activity (e g. liver and kidney of pig No I or brain and muscle tissue of all animals) are probably lacking such receptor sites. The above results stimulate detailed studies on the transfer mechanism of the A determinant from plasma to the cell membrane.

References

1 Thiele, O W Löslich Blutgruppenantigene von Glykolipidcharakter Blut 24 314–320 (1972).
Andresen, E. Blood groups i pigs. Ann. N. Y Acad. Sci. 97 205–225 (1962).
3 Stormont, C. Acquisition of the J substance by the bovine erythrocyte. Proc natn. Acad. Sci. USA 35 232 237 (1949).
4 Schröffel, J., Radal, A. Thiele, O W and Koch, J The J blood-group substance of cat

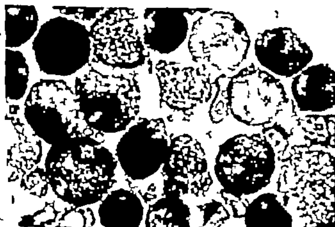


Fig. 1 Bone marrow smear showing many hypergranular promyelocytes × 1,100.

basin III zero. Liver function test were all within normal limits.

The bone marrow was impacted with immature cells and aspiration resulted in dry tap A trephine biopsy was performed and histopathology and touch imprints of the biopsy revealed massive infiltration with promyelocytes (fig 1), some of which contained Auer rods

Treatment with daunomycin was started platelet transfusions were administered Nevertheless, severe thrombocytopenia persisted and massive gastrointestinal bleeding continued constantly throughout the hospitalization Except for persistent thrombocytopenia repeated coagulation studies were all within normal limits, nd there was no laboratory evidence of consumption coagulopathy

On the 15th day of hospitalization, the patient developed septicemia and died.

Discussion

In nearly all cases of APL, it is the bleeding tendency that initially causes the patient to seek medical attention. The bleeding diathesis is generally more severe, than the bleeding encountered in other acute leukemias because disseminated intravascular coagulation (DIC) is present in most patients [3] However In our patient the bleeding was due to thrombocytopenia and there was no evidence for DIC.

In the absence of liver damage, the determination of serum vitamin B_{12} concentrations and levels of vitamin B_{12} binding proteins have been shown to be both reliable and useful aids in the diagnosis of APL [4]

Our patient did not achieve remission and died of septicemia 3 weeks after the diagnosis of APL was made. Since 1967 with the introduction of daunorubicin, APL has become a treatable disease and complete remission can be achieved in over half the patients with a median survival of 26 months [1] However as in our case, the cause of death in patients not in remission is invariably sepsis or massive hemorrhage

References

1 Bernard, J Weil, M., Boiron, M Jacquillat, C., Flandrin, G., and Gemon, M. F Acute promyelocytic leukemia. Result of treatment of daunorubicin. Blood *41* 489–496 (1973).

2 Flandrin, G and Bernard, J Cytological clas-

Short Communication

Acta haemat. 62: 282–284 (1979)

Acute Promyelocytic Leukemia in Childhood

Report of a Case with a Review of the Literature

Dan Engelhard, Shaul Yatziv, Eliezer A. Rachmilewitz and Aaron Polliack

Departments of Pediatrics and Hematology, Hadassah University Hospital and Hebrew University-Hadassah Medical School, Jerusalem

Key Words. Acute leukemia · Childhood leukemia · DIC · Promyelocyte

Abstract. A rare case of acute promyelocytic leukemia (APL) is reported in a 7-year-old boy. The patient displayed the typical features of APL including impaction of the marrow with promyelocytes, marked elevation of the serum vitamin B_{12} and transcobalamin I levels and a hemorrhagic diathesis. The bleeding diathesis in this case was due to *thrombocytopenia, and there was no evidence for disseminated intravascular coagulation.*

Acute promyelocytic leukemia (APL) is a rare variant of acute myeloblastic leukemia (AML) and constitutes approximately 3–5% of all acute leukemias [1, 2]. APL is rare in childhood, and of more than 125 cases reported until now, only 5 have been children under the age of 10 years [1, 3, 4]. In a review of close to 2,000 cases of acute childhood leukemia [5], APL was not even recognized as a separate entity.

During the past 5 years we have treated over 100 cases of children with acute leukemia and the patient reported here is the first case of APL that we have encountered. This patient had classical APL with a bleeding diathesis due to severe thrombocytopenia.

Case Report

A 7-year-old Arab boy was admitted to the Hadassah University Hospital, Jerusalem, after 2 days of melena and gingival bleeding.

On admission, physical examination revealed a pale, extremely ill child with a fever of 39 °C and severe melena. There were no ecchymoses or purpura. Laboratory investigations revealed severe anemia with a hemoglobin of 2.6 g%. The white cell count was 5,200/mm³ with 58% promyelocytes and the platelet count was 4,000/mm³. All other coagulation studies were within normal limits. Serum vitamin B and B binding proteins were grossly abnormal: serum vitamin B was 3,000 pg/ml, unsaturated vitamin B binding capacity 10,300 pg/ml, transcobalamin I 9,579 pg/ml, transcobalamin II 721 pg/ml, and transcoba-

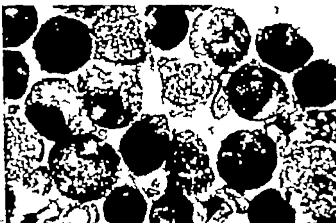


Fig. 1. Bone marrow smear showing many hypergranular promyelocytes. × 1 100.

tamin III zero. Liver function tests were all within normal limits.

The bone marrow was impacted with immature cells and aspiration resulted in dry tap. A trephine biopsy was performed and histopathology and touch imprints of the biopsy revealed massive infiltration with promyelocytes (fig. 1), some of which contained Auer rods.

Treatment with daunomycin as started, platelet transfusions were administered. Nevertheless, sever thrombocytopenia persisted and massive gastrointestinal bleeding continued constantly throughout the hospitalization. Except for persistent thrombocytopenia, repeated coagulation studies were all within normal limits, and there was no laboratory evidence of consumption coagulopathy.

On the 15th day of hospitalization, the patient developed septicemia and died.

Discussion

In nearly all cases of APL, it is the bleeding tendency that initially causes the patient to seek medical attention. The bleeding diathesis is generally more severe, than the bleeding encountered in other acute leukemias because disseminated intravascular coagulation (DIC) is present in most patients [3]. However, in our patient the bleeding was due to thrombocytopenia and there was no evidence for DIC.

In the absence of liver damage, the determination of serum vitamin B_{12} concentrations and levels of vitamin B_{12} binding proteins have been shown to be both reliable and useful aids in the diagnosis of APL [4].

Our patient did not achieve remission and died of septicemia 3 weeks after the diagnosis of APL was made. Since 1967 with the introduction of daunorubicin, APL has become a treatable disease and complete remission can be achieved in over half the patients with a median survival of 26 months [1]. However, as in our case, the cause of death in patients not in remission is invariably sepsis or massive hemorrhage.

References

1 Bernard, J.; Weil, M.; Boiron, M.; Jacquillat, C.; Flandrin, G., and Gemon, M. F.: Acute promyelocytic leukemia. Results of treatment of daunorubicin. Blood 41: 489–496 (1973).

2 Flandrin, G. and Bernard, J.: Cytological clas-

Acta haemat. 62 282–284 (1979)

Acute Promyelocytic Leukemia in Childhood

Report of a Case with a Review of the Literature

Dan Engelhard Shaul Yatziv Eliezer A Rachmilewitz and Aaron Polliack

Departments of Pediatrics and Hematology Hadassah University Hospital and Hebrew University Hadassah Medical School, Jerusalem

Key Words. Acute leukemia Childhood leukemia DIC Promyelocyte

Abstract. A rare case of acute promyelocytic leukemia (APL) is reported in a 7-year old boy The patient displayed the typical features of APL including impaction of the marrow with promyelocytes, marked elevation of the serum vitamin B_{12} and transcobalamin I levels and a hemorrhagic diathesis. The bleeding diathesis in this case was due to thrombocytopenia, and there was no evidence for disseminated intravascular coagulation.

Acute promyelocytic leukemia (APL) is a rare variant of acute myeloblastic leukemia (AML) and constitutes approximately 3–5% of all acute leukemias [1 2] APL is rare in childhood, and of more than 125 cases reported until now only 5 have been children under the age of 10 years [1 3 4] In a review of close to 2,000 cases of acute childhood leukemia [5] APL was not even recognized as a separate entity

During the past 5 years we have treated over 100 cases of children with acute leukemia and the patient reported here is the first case of APL that we have encountered. This patient had classical APL with a bleeding diathesis due to severe thrombocytopenia

Case Report

A 7 year-old Arab boy was admitted to the Hadassah University Hospital, Jerusalem, after days of melena and gingival bleeding.

On admission, physical examination revealed a pale, extremely ill child with a fever of 39 C and severe melena. There were no ecchymoses of purpura. Laboratory investigations revealed severe anemia with a hemoglobin of 2.6 g% . The white cell count was 5,200/mm with 58% promyelocytes and the platelet count was 4,000 mm³ All other coagulation studies were within normal limits. Serum vitamin B and B binding proteins were grossly abnormal serum vitamin B was 3,000 pg/ml, unsaturated vitamin B binding capacity 10,300 pg/ml, transcobalamin I 9,579 pg/ml, transcobalamin II 721 pg/ml, and transcoba-

Proceedings of a Symposium
held in Marburg a. d. Lahn
March 15 1979

Cultivation of Haematopoietic Stem Cells and of Committed Leukocyte Progenitor Cells

Editors
W. D. Gassel and K. Havemann,
Marburg a. d. Lahn

20 figures, 3 tables, 1979



S. Karger Basel München Paris London New York Sydney

sification of acute leukemias. a survey of 1 400 cases. Blood Cells *1* 7–15 (1975).

3 Gralnick, H R. Bargley J and Abrell, E. Heparin treatment for the hemorrhagic diathesis of acute promyelocytic leukemia. Am. J Med. *52*. 167–174 (1972)

4 Rachmilewitz, D Rachmilewitz, E. A., Polliack, A., and Hershko, C., Acute promyelocytic leukemia. *a report of five cases with a comment on the diagnostic significance of serum vitamin B ₁₂ determination* Br J Haemat. *22* 87–92 (1972).

5 Pierce, J L., Borges, W H. Heyn, R. Wolff J A., and Gilbert, E. S. Epidemiological factors and survival experience in 1 770 children with acute leukemia. Cancer *23* 1296–1304 (1969).

Received February 8, 1979
Accepted May 29 1979

Prof. Aaron Polliack, MD
Department of Hematology
Hadassah University Hospital,
Jerusalem (Israel)

Contents

S. Karger AG P O Box 4009 Basel (Switzerland)
Printed in Switzerland by Basler Zeitung, Basel

Acta haemat. 62: 289–298 (1979)

Light Scattering Properties of Pluripotent and Committed Haemopoietic Stem Cells

Ger van den Engh and Jan Visser

Radiobiological Institute TNO Rijswijk, and Department of Radiobiology, Medical Faculty Erasmus University, Rotterdam

Key Words. Cell sorter · CFU-c · CFU-s · Haemopoiesis · Light scatter

Abstract. Mouse bone marrow cells were separated according to their light scattering properties in a light activated cell sorter. The intensity of the scattered light was measured in a forward direction (FLS) and perpendicular to the incident light (PLS). These two parameters were found to give an indication of cell size and shape. The fractions containing cells with different light scattering properties were assayed for CFU-s (pluripotent stem cells) content and for the incidence of three types of CFU-c (cells committed to granulocyte/macrophage differentiation). These measurements confirm previous size calculations based on sedimentation rate and density separation. During maturation, the different colony forming units (CFU) show a gradual increase in PLS intensity. This suggests that the transition from CFU-s through to CFU-c 3 is accompanied by a morphological differentiation.

Introduction

In the study of haemopoiesis one often makes use of *in vivo* and *in vitro* cloning assays in which pluripotent stem cells and cells committed to granulocyte/macrophage differentiation are detected. The need to perform colony assays makes it necessary that cell viability is preserved throughout the experiments. This limits the isolation procedures that can be employed to concentrate the rare stem cells from the complex mixture of cell types which is found in the haemopoietic organs. Cell separation techniques were developed in which physical properties of the cells, such as cell density or sedimentation rate, can be determined without affecting cell viability [*Shortman*, 1972; *Miller*, 1973].

Studies using these techniques indicate that the haemopoietic cells form a heterogeneous group. The cells which give rise to spleen colonies *in vivo* (spleen colony-forming units, CFU-s) are considered to represent pluripotent haemopoietic stem cells. They may generate offspring of all the blood lineages [*Till and McCulloch*, 1961]. The pluripotent CFU-s can be physically separated from three types of *in vitro* colony-forming cells (CFU-c 1, 2 and 3) which

and was kept at low temperatures throughout the experiments. Thymocyte suspensions were obtained by gently disrupting thymi in the same buffer. Shortly before analyzing and sorting by the fluorescence-activated cell sorter (FACS), cells were resuspended in phosphate-buffered saline (PBS) of 300–310 mO sm (pH 6.7).

CFU-s Assay

Mice (8–12 weeks of age) were irradiated with 1,025 rad γ-irradiation and injected on the same day with marrow cells obtained from the same mouse strain. In each experiment control group of mice not receiving any bone marrow cells was incorporated. The mice were housed 5 to cage and were fed sterile food and acid water. Spleen colonies were counted 9 days after irradiation [*Till and McCulloch*, 1961]. The spleens of the mice of the control group generally did not contain colonies.

In vitro Colony F rmation

CFU-c numbers were determined in an assay which is slight modification of the one developed by *Bradley and Metcalf* [1966]. Details are published elsewhere [*van den Engh*, 1974 *van den Engh and Bol*, 1975]. The culture dishes contain 1 ml cell suspension in 0.3% agar and Dulbecco's medium with 5×10^4 bone marrow cells. To obtain proliferation of granulocyte/macrophage progenitor cells growth stimulator (CSF) has to be added. In all cultures, CSF from pregnant mouse uteri was used. The cell which forms colonies with this type of CSF has been designated as CFU-c 2 *Bol et al.*, 1979]. When serum from endotoxin-treated mice is added to such cultures, an earlier CFU-c type (CFU-c 1) will form additional colonies. Addition of erythrocyte lysate gives colonies from yet another CFU-c type (CFU-c 3), which is the most mature of the three. The *in vitro* colony-forming cells have been termed CFU-c 1, 2 and 3 in the sequence of maturation (fig. 1).

Light-Activated Cell Sorting

The FACS II cell sorter (Becton and Dickinson, Mountain View Calif.) was used. FLS was measured on the small angle scatter diode with fully opened diaphragm. Over-exposure of the diode was prevented by neutral density filter with transmission of 2.5% The laser stopping

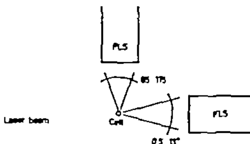


Fig. 2. Angles of acceptance of the FLS and PLS detectors in a FACS II cell sorter. The laser light blocking bar is set as flat as possible and the diaphragm is fully opened.

bar was adjusted as narrow as possible. In the set up the angle of acceptance was 0.5–13° with respect to the direction of the laser beam (fig. 2). The fluorescence detector was used to measure PLS. For this purpose filters blocking the laser light were removed. With the diaphragm fully opened, the detector covered an angle of 65–115° with respect to the laser beam (fig. 2).

The cell sample was injected in sheath flow of PBS. The collection tubes contained 25% calf serum. The sides of the tubes were rinsed befor and after sorting to prevent cells from sticking to the sides. The cell sorter was sterilized by flushing the tubing with sterile glass distilled water and 5% hibitane in 70% ethanol, followed by final rinse of sterile H_2O

Staining of Cells with Propidium Iodide

In order to stain the DNA of dead cells, small amounts of propidium iodide (final concentration 5 μg/ml) were added to the cell samples. Dead cells could be recognized on the basis of their red fluorescence. The same method was used to measure the DNA content of sorted thymocytes.

Results

Interpretation of Light Scatter Measurements

Light hitting a small translucent particle is mainly scattered into the forward direc

are committed to maturation into granulocytes and/or macrophages [*Williams and van den Engh* 1975 *Bol et al* 1979] These *in vitro* colony-forming cells depend on the addition of growth stimulators to the culture in order to be able to proliferate. Which type of CFU-c is stimulated depends on the presence of co-factors. The three CFU-c types represent three subsequent stages of maturation which are characterized by increasing cell density [*Bol and Williams* in press] Although differences in density of the CFU types can be demonstrated, they are small compared to the total density range spanned by all blood cells A scheme of the pathway from CFU-s to the most mature CFU-c 3 is shown in figure 1

In this paper experiments with a new method of viable cell separation are reported. The optical properties of the CFU are determined in a light-activated cell sorter [*Herzenberg et al.* 1976] In a cell sorter cells are passed through a laser beam in a liquid jet in air This is usually done to measure fluorescence of stained cells [*Horan and Wheeless* 1977 *Herzenberg and Herzenberg* 1978] The cells also cause light scatter as they traverse the laser light [*Salzman et al* 1975 *Loken et al* 1976 *Jovin et al* 1976] By measuring the light scatter in two directions, information about cell size and structure is obtained. Light scatter intensity is a useful criterium for distinguishing the different cell types in bone marrow [*Loken et al.*, 1976 *Salzman et al.*, 1975 *Visser et al.*, 1978a] The size determination of CFU based on forward light scatter (FLS) measurement confirms those previously derived from sedimentation rate and density separation. Measurement of the light scatter intensity in a perpendicular direction shows that the earliest CFU have a spherical shape with little internal structure. CFU-c 2 and 3 cause increasing perpendicular light scatter (PLS) signals, indicating that morphological changes take place during these early differentiation steps.

Material and Methods

Cell Suspensions

The bone marrow cells were obtained from C3H or F1(C3H × C57BL) mice. The femora were flushed with Hanks' salt solution containing HEPES buffer with 10% fetal calf serum. The cell suspension was immediately put on melting ice

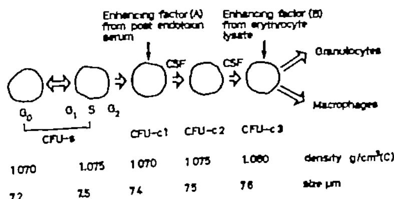
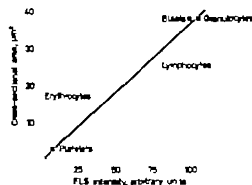


Fig. 1. Schematic representation of the earliest stages of granulocyte/macrophage differentiation. The pluripotent haemopoietic stem cells (CFU-s) give rise to committed cells (CFU-c) which are capable of colony formation *in vitro* Depending on the culture conditions, three types of CFU-c can be induced to form colonies.

A = *van den Engh and Bol* [1975]· B = *Williams and van den Engh* [1975] C = *Bol et al.* [1979].



fore be high for non-spherical cells, cells with non-spherical internal structures (granulocytes/erythrocytes) or cells with internal particles of a high refractive index (granulocytes). The validity of this assumption is demonstrated in figure 5. It shows a dot display of PLS intensity versus FLS intensity

Fig. 4. Relative FLS intensity of different mouse cell types versus their cross-sectional area. The cross-sectional area is divided from sedimentation rate and density measurements.

Thymocytes



PLS, arbitrary units

Bone marrow cells

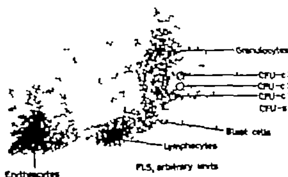


Fig. 5. Dot display of FLS versus PLS of mouse thymocytes (top) and bone marrow cells (bottom). Each dot represents the relative FLS and PLS signal from one cell.

tion [*van de Hulst* 1957 *Kerker* 1969] This FLS can be largely attributed to diffraction and refraction of the light. If the FLS detector of the FACS II cell sorter is adjusted so that the angle of acceptance is as wide as possible (as is the case in these experiments) the FLS detector will collect most of the scattered light. Since the total light scattered is equal to the amount of light intercepted by the particle, the intensity of the light measured on the FLS detector can be expected to be approximately proportional to the cross-sectional area of the particle. Using fluorescent Sephadex spheres, *Visser et al* [1978b] showed that this relationship holds for uniform dextran spheres. Similar results were obtained in measurements of mouse erythrocytes at varying osmolarities [*Visser et al* in press]

The following two examples demonstrate that the cross sectional area rule may also serve as an approximation for the FLS signals of living mouse leukocytes. The top panel of figure 3 shows the FLS intensity distribution of mouse thymocytes. In a logarithmic representation of the histogram (fig. 3 bottom) it is seen that the cell frequency rapidly decreases at channel numbers higher than 1.59 times the peak value. This can be ascribed to the fact that cells in G_2 have twice the volume of cells in G_1. Hence spherical cells differ by a factor of 1.59 in the cross sectional area. To test the assumption that the boundaries of the FLS distribution of thymocytes show G_1 and G_2 cells, cells from the two extreme regions were sorted and stained for DNA content with propidium iodide. The amplification of the FLS signals was adjusted so that the peak fell in channel 100. A subsequent run showed that the cells in channel 100 had a DNA content of 2 n and that the large majority of cells found around channel 160 had twice as much DNA.

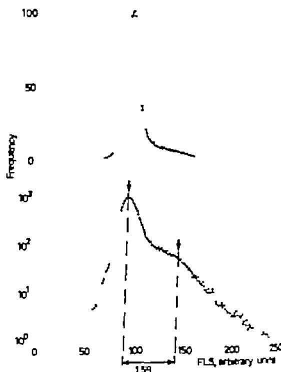


Fig. 3. FLS distribution of mouse thymocytes. The bottom panel is a logarithmic representation of the data shown in the top panel.

The cross-sectional area of cells can also be calculated from velocity rate and density measurements. There is a good agreement between cross-sectional area of mouse blood cells which is calculated in this manner and FLS intensity measurements (fig. 4). These observations indicate that FLS intensity of a cell is a good approximation for its cross-sectional area.

The light scattered perpendicular to the direction laser beam is only slightly affected by cell size. This *PLS is* due to refraction and reflection of the laser light. The contribution of these two phenomena are low for spherical shapes and increase in importance with an increasing surface/volume ratio of the particle. The PLS intensity will there-

fore be high for non-spherical cells, cells with non-spherical internal structures (granulocytes/erythrocytes) or cells with internal particles of a high refractive index (granulocytes). The validity of this assumption is demonstrated in figure 5. It shows a dot display of PLS intensity versus FLS intensity

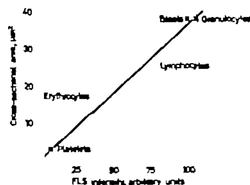


Fig. 4. Relative FLS intensity of different mouse cell types versus their cross-sectional area. The cross-sectional area is divided from sedimentation rate and density measurements.



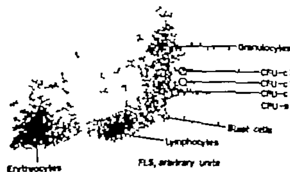


Fig. 5. Dot display of FLS versus PLS of mouse thymocytes (top) and bone marrow cells (bottom). Each dot represents the relative FLS and PLS signal from one cell.

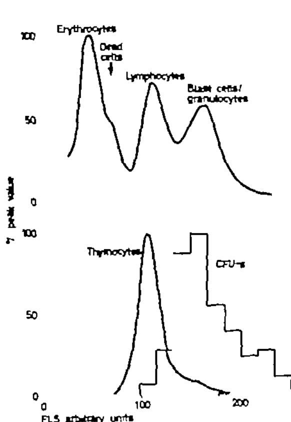


Fig. 6. FLS distributions of mouse thymocytes, bone marrow cells and CFU-s.

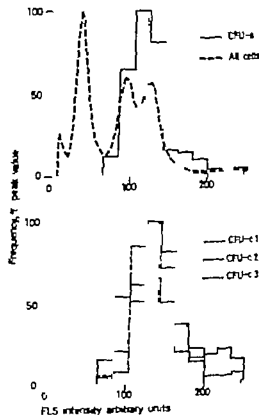


Fig. 7 The FLS distributions of mouse bone marrow cells, CFU-s and three CFU-c types. The median of the distribution was calculated for each of the CFU

of mouse thymocytes and mouse bone marrow cells. The cells belonging to the various clusters were sorted on glass slides, fixed and stained by routine haematological methods and identified through a microscope. The relation between FLS and PLS intensity confirms the theoretical considerations. FLS intensity increases with cell size whereas the PLS intensities are high for non-spherical cells. Since the size of the thymocytes at the peak of the distribution is known (5 9 μm in diameter derived from sedimentation rate and density data of Boersma) the intensity of the FLS signals can be calibrated with respect to cell size.

Light Scattering Properties of CFU s and CFU-c

The FLS properties of CFU-s and the CFU-c types were determined by separating mouse bone marrow cells into fractions of increasing FLS intensity The FLS intensity distributions of all bone marrow cells and thymocytes were also measured. Cell fractions were injected into mice for CFU-s determination. The cells were also cultured *in vitro* with different combinations of stimulating factors. The recovery of the cell types was in the order of 70–90%.

The distributions of CFU-s and CFU-c over the FLS profile are shown in figures 6

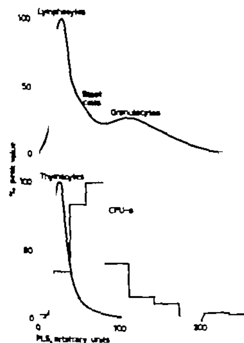


Fig. 8. The PLS distributions of mouse thymocytes, bone marrow cells and CFU-s.

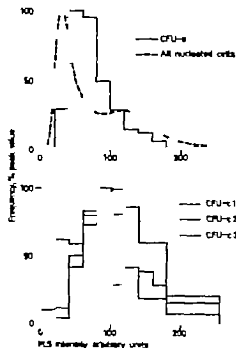


Fig. 9 The PLS distributions of mouse bone marrow cells, CFU-s and three CFU-c types. The median of the distribution was calculated for each of the CFU

and 7 Since the steps are too coarse to assign an accurate peak value to the distributions, the medians of the curves were taken as a reference value Since cycling cells have a skew size distribution, the place of this value will be slightly higher than the peak of G cells. The position of the median will also depend on the proliferative status of the cells. The intensities of FLS of the three CFU-c types are very similar The FLS intensity of CFU-s is somewhat lower In the experiment of figure 7 the median of the CFU-s distribution coincides with channel 117 The medians of CFU-c 1 2 and 3 are found in channels 133 137 and 137 respectively From figure 6 it can be derived that the median of CFU-s is found at approximately 1.5 times the channel in which the peak of the thymocyte FLS distribution is found.

Similar experiments were performed using the PLS intensity of cells as a separation criterium (fig. 8 9). In this case, a clear separation of CFU-s and CFU-c 1 2 and 3 was obtained. The medians of the four cell populations were found in channels 69 86, 105 and 120, respectively

The coordinates of the medians of FLS and PLS scatter are plotted in figure 5 If the ratio of PLS to FLS of G thymocytes is taken as 1 the ratio of CFU-s becomes 1 7 that of CFU-c 1 1.9 that of CFU-c 2 2.2

and that of CFU-c 3 2.6 Since this ratio is minimal for spherical cells, the gradual increase suggests a morphological development from CFU-s to CFU-c 3

Discussion

The experiments show that stem cells from haemopoietic cells can be analyzed in a light-activated sorter and can be electrostatically sorted into test tubes without loss of cell viability Throughout the experiments recovery of CFU-s and CFU-c was in the order of 70–100%.

The intensities of FLS and PLS proved to be convenient criteria for recognizing subpopulations of blood cells FLS as measured in the FACS II with fully opened diaphragm is a measure of cell size (proportional to cross-sectional area of the cell) The PLS intensity is a measure of the degree of folding of the cell and nucleus membrane and/or the presence of inclusions in the cytoplasm Using these two criteria, a 5-fold increase in CFU-s concentration can be reached. Since these measurements require no pretreatment of the cells, the enrichment can be combined with the majority of experiments done on a cell sorter For instance, determination of surface antigens of haemopoietic stem cells with fluorescent antibody are facilitated if only the cells in the CFU-s area are considered.

The FLS and PLS intensity measurements also give information on the morphology of the cell types. The morphological structure of these cells is reflected in their optical properties. In this sense, cell sorters offer a unique possibility to determine morphological characteristics of cells without staining, with maintenance of cell viability and with the option to obtain the cell in a purified state. With the ongoing development of more precise multiangle scatter detectors and improving understanding of scatter signals from cells [*Kerker et al.* 1979 *Genter and Salzman*, 1979] the morphological information that can be derived can be expected to increase considerably

The relatively simple two-direction wide angle measurements reported here allow determination of CFU size and give a rough indication of CFU morphology The median of the CFU-s distribution was found to have a FLS intensity 1.5 times the peak of the thymocyte FLS distribution. According to the cross-sectional area relationship, CFU-s are estimated to measure 7.2 μm in diameter The size calculation of CFU-c 1 is 7 7 μm. Since the median of the FLS distribution is used rather than the peak value of the distribution, these estimations will be somewhat larger than the G_1 size. Considering this, the data are in good agreement with the CFU sizes previously reported (fig. 1). The two other *in vitro* colony-forming cells, CFU-c 2 and 3 are similar in size to CFU-c 1 but have an increasing PLS intensity This suggests that gradual morphological changes take place in the pathway from pluripotent CFU-s to the committed CFU-c 3 This confirms the maturation sequence for CFU-c proposed by *Bol and Williams* (in press).

Previous conclusions that *in vitro* colony-forming cells are a heterogeneous group of cells and that different sources may stimulate different CFU-c types in mixtures of CFU-c are confirmed by this study The three groups of CFU-c which differ in density are also found to be different in PLS distribution Since the FLS signals of three

CFU-c do not significantly differ in intensity the previous conclusion that the heterogeneity in sedimentation rate is largely due to cell density and, that differences in cell size are of minor importance is also confirmed [*Bol et al* 1979]

Acknowledgements

This investigation is part of project program on the regulation of haemopoiesis, which is supported by the Netherlands Foundation for Medical Research (FUNGO).

References

Bol, S. J. L., Visser J. W. M., and Engh, G. J. van den: The physical separation of three subpopulations of granulocyte/macrophage progenitor cells from mouse bone marrow Exp. Hematol (in press, 1979).

Bol, S. J. L. and Williams, N. T. The maturation state of three types of granulocyte/macrophage progenitor cells from mouse bone marrow J Cell. Physiol. (in press)

Bradley T R. and Metcalf, D. The growth of mouse bone marrow cells *in vitro* Aust. J exp Biol. med. Sci *44* 287 (1966).

Engh, G. J. van den. Quantitative *in vitro* studies on stimulation of murine haemopoietic cells by colony-stimulation factor Cell Tiss Kinet. 7 537 (1974)

Engh, G. J. van den and Bol, S. J. L. The presence of CSF enhancing activity in the serum of endotoxin-treated mice Cell Tiss Kinet *8* 579 (1975)

Genter F. C. and Salzman, G. C. A statistical approach to the classification of biological cells from their diffraction patterns. J Histochem. Cytochem *27* 268–272 (1979).

Herzenberg, L. A. and Herzenberg, L. A.: Analysis and separation using the fluorescence-activated cell sorter (FACS) in Weir, Handbook of experimental immunology chapter 22 (Blackwell, Oxford 1978).

Herzenberg, L. A., Sweet, R. G. and Herzenberg, L. A. Fluorescence-activated cell sorting. Scient. Am. *234* 108–117 (1976).

Horan, P. K. and Wheeless, L. L., jr Quantitative single cell analysis of sorting. Science *198*. 149–157 (1977).

Hulst, H. C. van de: Light scattering by small particles (Wiley New York 1957).

Jovin, T. M. Morris, S. J. Striker G. Schultens, H. A. Digweed, M., and Arndt-Jovin, D. J. Automatic sizing and separation of particles by ratios of light scattering intensities. J Histochem. Cytochem. *24* 269–283 (1976).

Kerker M. The scattering of light and other electromagnetic radiation (Academic Press, New York 1979).

Kerker M., Chew H., McNulty P. J. Kratohvil, J. P. Cooke, D. D. M., Sculley M., and Lee, M. P. Light scattering and fluorescence by small particles having internal structure J Histochem. Cytochem. 27 250–263 (1979).

Loken, M. R., Sweet, R. G., and Herzenberg, C. A. Cell discrimination by multiangle light scattering. J Histochem. Cytochem. *24* 284–291 (1976).

Miller R. G. Separation of cells by velocity sedimentation in Pain and Smith, New techniques in biophysics and cell biology (Wiley London 1973).

Mullaney P. F. Dilla, M. A. van; Coulter J. R., and Dean, P. N., Cell sizing: light scattering photometer for rapid volume determination. Rev scient Instrum. 40· 1029 (1969).

Salzman, G. C., Crowell, J. M., Martin, J. C.; Trujillo, T. T. Romero, A., Mullaney P. F. and LaBauve, P. M. Cell classification by laser light scattering: identification and separation of unstained leukocytes. Acta cytol. *19*· 374–377 (1975).

Shortman, K. Physical procedures for separation of animal cells. Annu. Rev Biophys. Bioeng. *1* 93 (1972).

Till, J. E. and McCulloch, E. A. A direct measurement of the radiation sensitivity of normal mouse bone marrow cells. Radiat. Res. *14* 213 (1961).

Visser J. W. M.; Cram, L. S. Martin, J. C., Salzman, G. C., and Price, B. J. Sorting of murine granulocytic progenitor cell by use of laser light scattering measurements, in Lutz, Pul-

se-cytophotometry vol. III pp. 187–192 (European Press, Ghent 1978a).

Visser J W M Haaijman, J and Trask, B J.: Quantitative immunofluorescence in flow cytometry in Knapp, Holubar and Wick, Immunofluorescence and related staining techniques (Elsevier/North Holland, Amsterdam 1978b)

Visser J W M., Engh, G J van den, and Bekkum, D W van Light scattering properties of murine haemopoietic cells. Blood Cells (in press).

Williams, N T and Engh, G J van den: Separation of subpopulations of *in vitro* colony-forming cells from mouse marrow by equilibrium density centrifugation. J cell. Physiol. 86. 237 (1975)

Ger van den Engh,
Radiobiological Institute TNO
151 Lange Kleiweg,
Rijswijk (The Netherlands)

Acta haemat. 62, 299–305 (1979)

Haemopoiesis in Long-Term Bone Marrow Cultures

A Review

T. M. Dexter

Paterson Laboratories, Christie Hospital and Holt Radium Institute, Manchester

Key Words. Long-term cultures · Stem cell · Proliferation · Differentiation · Transplantation

Abstract. Bone marrow-derived adherent cell layers, containing endothelial cells, fat cells and macrophages will support prolonged haemopoiesis *in vitro*. Evidence suggests that the adherent layer is acting as an *in vitro* haemopoietic inductive environment for stem cell proliferation and differentiation into the variety of committed progenitor cells of the granulocytic, erythroid, megakaryocytic and lymphoid lineages. Using this system we have analysed the factors controlling proliferation of stem cells (CFU-S), differentiation and maturation of granulocyte/macrophage precursor cells (CFU-C), leukaemic transformation by chemicals and viruses, the role of environment versus stem cells in the aetiology of haemopoietic aplasias and the possible usefulness of long-term cultures for bone marrow transplantation.

Introduction

Almost all of our knowledge on the factors controlling haemopoiesis is concentrated on the terminal aspects of differentiation and maturation. For example, it is generally accepted that the development of granulocyte precursor cells *in vitro* (CFU-C) depends upon the continued presence of appropriate colony-stimulating factor(s) (CSF), which have been characterised in terms of their heterogeneity, dose-response characteristics and even to the isolation of apparently pure molecular species [22]. Similarly, the role of lactoferrin in inhibiting the production of CSF [3] and of the prostaglandin E series in altering the responsiveness of CFU-C to CSF molecules [18] has been elegantly established. Moreover, several groups are actively concerned with analysis of the variety of molecules produced from stimulated lymphocyte and macrophage populations and their role in granulocyte/macrophage maturation.

In another system, there is the role of burst-promoting activity (BPA) in the progression of the early *in vitro* erythroid progenitor cells (BFU-E) into cells which are

receptive to the hormone erythropoietin and other molecules necessary for haemoglobinisation to occur [17]. Thus far little is known of the regulation of megakaryopoiesis – although potential humoral factors are presently being characterised, and colony stimulation molecules necessary for *in vitro* growth of megakaryocyte precursor cells are being isolated [21, 22, 28]. Finally the role of antigens, mitogens, growth-promoting activities of macrophages and possible humoral factors such as thymopoietin [15] are being investigated in T and B lymphocyte development.

The list is by no means exhaustive, but does serve to show the range of studies being conducted on these more differentiated cell populations and also underlines the major gap in our knowledge – namely the control of the pluripotent stem cell population. In recent years, we have developed a culture system [4–7] whereby haemopoietic stem cell proliferation and differentiation can be maintained for several months, and which seems to be a suitable model system for studying the regulatory molecules and cellular interactions involved in this earliest compartment.

In this communication I would like to review some of the studies performed with these long-term cultures and the application of the technique to the study of haemopoietic dysplasia, *in vitro* leukaemogenesis and proliferation/differentiation control mechanisms.

Establishing the Cultures

To establish the cultures, the contents of a mouse femur are flushed into culture flasks (Corning, 25 cm^2) containing 10 ml of Fischer's medium (Gibco) and 25% horse serum (Flow Laboratories) supplemented with antibiotics as previously described [4]. No attempt is made to obtain a single cell suspension. Many batches of horse serum and all batches of fetal calf serum were found to be deficient in their capacity to maintain *in vitro* haemopoiesis, but can be successfully reconstituted by the addition of hydrocortisone as described by *Greenberger* [16] and confirmed by us [unpublished].

The cultures are maintained in air + 5% CO_2 at a temperature of 33 °C [7] and are fed weekly by removal of half the growth medium and addition of an equal volume of fresh medium. Over a 2- to 3-week period, an adherent layer of cells is formed (see later) and the cultures are then re-inoculated with a further population of 5×10^6–10^7 syngeneic or allogeneic marrow cells [6, 7, 12]. These constitute the cultures proper and are fed weekly by demi-depopulation as described earlier. The growth medium removed contains cells in suspension which, after appropriate concentration or dilution, can be assayed for the haemopoietic stem cells (CFU-S) [26] or the variety of committed progenitor cells. In this way events occurring in individual cultures can be monitored over many weeks.

The Non-Adherent Cells

These cells show an approximate doubling after each 'feed' and consist mainly of granulocytes in all stages of maturation [7, 11]. Megakaryocytes are also being produced, but the cultures contain no morphologically recognisable erythroid cells or lymphocytes [2, 7]. Pluripotent stem cells (CFU-S) are being produced for periods of 10–18

weeks, again showing an approximate doubling between each feed. Such stem cells are functionally quite normal [7] forming all haemopoietic elements and protecting mice from potentially lethal radiation when injected *in vivo*. Along with stem cell proliferation there is a production of CFU-C [7, 27] BFU-E [25] and megakaryocyte precursor cells [28]. Recent evidence also indicates the prolonged maintenance of a committed lymphoid stem cell (restricted to B and T lymphopoiesis) [23] and of a 'pre T' lymphoid cell population [*Phillips*, personal commun. 1978]. Therefore, the cultures produce the whole range of differentiated progeny for many weeks, although, within the cultures, further differentiation and maturation is limited to the granulocyte and megakaryocyte lineages.

The Adherent Cells

In the absence of an adherent layer stem cells rapidly decline and disappear within a few days. Conclusive evidence for the role of the adherent cell population in the long-term cultures comes from our studies with genetically anaemic mutant mice [8]. Two strains are of importance: Sl/Sl[d] mice which have a defective inductive environment (but normal stem cells) and W/W[v] mice which have an intrinsic defect in the stem cell population (although the inductive environment is quite normal). Using the long-term cultures, we showed that these defects can be reproduced *in vitro* [8] and that they can be overcome using the appropriate combination of adherent layer and stem cells. For example, adherent layers from Sl/Sl mice (defective) re-inoculated with marrow stem cells from W/W[v] mice (defective) showed no maintenance of haemopoiesis, whereas the reverse combination (W/W[v] re-inoculated with Sl/Sl[d]) showed a prolonged *in vitro* haemopoiesis. The conclusion was that stem cell proliferation *in vitro* depends upon interactions between a competent stem cell population and the appropriate inductive environment.

This begs the question as to the cell population(s) which make up the adherent layer. Careful ultrastructural analysis has shown that at least three cell populations are present – which are probably representative of marrow stromal elements *in vivo* [1, 2, 7, 11]. These *in vitro* populations are made up of cells forming a flattened pavement-like structure (which we speculate are derived from the endothelium of the marrow sinus), macrophages and fat cells. The three cell types are often found in intimate association as a complex cellular multilayer [1, 2] often enclosing areas of active haemopoiesis.

The nature of the adherent layer suggested to us that the commitment for stem cell proliferation and differentiation arises from cellular interactions occurring in the adherent layer. Certainly the adherent layer is a major *site* of stem cell production [5, 10] and regularly regenerates the stem cells removed from the 'non-adherent' cell population consequent upon feeding the cultures.

Stimulus for Stem Cell Proliferation

We noticed some years ago that the stimulus for stem cell proliferation seemed to be associated with the feeding regime employed [10]. Thus, within 24 h following a 'feed' of the cultures, stem cells present both in the growth medium and in the adherent cell population are in an actively cy-

receptive to the hormone erythropoietin and other molecules necessary for haemoglobinisation to occur [17] Thus far little is known of the regulation of megakaryopoiesis – although potential humoral factors are presently being characterised, and colony stimulation molecules necessary for *in vitro* growth of megakaryocyte precursor cells are being isolated [21 22, 28] Finally the role of antigens mitogens, growth-promoting activities of macrophages and possible humoral factors such as thymopoietin [15] are being investigated in T and B lymphocyte development.

The list is by no means exhaustive but does serve to show the range of studies being conducted on these more differentiated cell populations and also underlines the major gap in our knowledge – namely the control of the pluripotent stem cell population In recent years, we have developed a culture system [4 7] whereby haemopoietic stem cell proliferation and differentiation can be maintained for several months, and which seems to be a suitable model system for studying the regulatory molecules and cellular interactions involved in this earliest compartment.

In this communication I would like to review some of the studies performed with these long-term cultures and the application of the technique to the study of haemopoietic dysplasia, *in vitro* leukaemogenesis and proliferation/differentiation control mechanisms.

Establishing the Cultures

To establish the cultures the contents of a mouse femur are flushed into culture flasks (Corning, 25 cm²) containing 10 ml of Fischer's medium (Gibco) and 25% horse serum (Flow Laboratories) supplemented with antibiotics as previously described [4]. No attempt is made to obtain a single cell suspension. Many batches of horse serum and all batches of fetal calf serum were found to be deficient in their capacity to maintain *in vitro* haemopoiesis, but can be successfully 'reconstituted' by the addition of hydrocortisone as described by Greenberger [16] and confirmed by us [unpublished]

The cultures are maintained in air + 5% CO_2 at a temperature of 33 C [7] and are fed weekly by removal of half the growth medium and addition of an equal volume of fresh medium. Over a 2 to 3-week period, an adherent layer of cells is formed (see later) and the cultures are then re-inoculated with a further population of 5×10^6–10^7 syngeneic or allogeneic marrow cells [6, 7 12] These constitute the cultures proper and are fed weekly by demi-depopulation as described earlier The growth medium removed contains cells in suspension which, after appropriate concentration or dilution, can be assayed for the haemopoietic stem cells (CFU-S) [26] or the variety of committed progenitor cells. In this way events occurring in individual cultures can be monitored over many weeks.

The Non-Adherent Cells

These cells show an approximate doubling after each 'feed' and consist mainly of granulocytes in all stages of maturation [7 11] Megakaryocytes are also being produced, but the cultures contain no morphologically recognisable erythroid cells or lymphocytes [2, 7] Pluripotent stem cells (CFU-S) are being produced for periods of 10–18

eeks, again showing an approximate doubling between each feed. Such stem cells are 'unctionally quite normal [7] forming all elements and protecting mice rom potentially lethal radiation when in ected *in vivo* Along with stem cell prolif there is a production of CFU-C / 27] BFU-E [25] and megakaryocyte cells [28] Recent evidence also the prolonged maintenance of a 'committed lymphoid 'stem cell (restricted 'o B and T lymphopoiesis) [23] and of a pre T' lymphoid cell population [*Phillips* I commun. 1978] Therefore the tures produce the whole range of differ entiated progeny for many weeks, although, ithin the cultures, further differentiation nd maturation is limited to the granulocyte nd megakaryocyte lineages.

The Adherent Cells

In the absence of an adherent layer stem 's rapidly decline and disappear within a v days. Conclusive evidence for the role f the adherent cell population in the long-term cultures comes from our studies with 'ly anaemic mutant mice [8] Two are of importance: Sl/Sl[d] mice which have a defective inductive environment (but normal stem cells) and W/W[v] ice which have an intrinsic defect in the em cell population (although the inductive is quite normal). Using the term cultures, we showed that these fects can be reproduced *in vitro* [8] and hat they can be overcome using the appropriate combination of adherent layer and cells. For example, adherent layers rom Sl/Sl[d] mice (defective) re-inoculated ith marrow stem cells from W/W[v] mice (defective) showed no maintenance of haemopoiesis, whereas the reverse combination (W/W[v] re-inoculated with Sl/Sl[d]) showed a prolonged *in vitro* haemopoiesis. The conclusion was that stem cell proliferation *in vitro* depends upon interactions between a competent stem cell population and the appropriate inductive environment.

This begs the question as to the cell population(s) which make up the adherent layer Careful ultrastructural analysis has shown that at least three cell populations are present – which are probably representative of marrow stromal elements *in vivo* [1 2, 7 11] These *in vitro* populations are made up of cells forming a flattened pavement-like structure (which we speculate are derived from the endothelium of the marrow sinus), macrophages and fat cells. The three cell types are often found in intimate association as a complex cellular multilayer [1 2] often enclosing areas of active haemopoiesis.

The nature of the adherent layer suggested to us that the commitment for stem cell proliferation and differentiation arises from cellular interactions occurring in the adherent layer Certainly the adherent layer is a major *site* of stem cell production [5 10] and regularly regenerates the stem cells removed from the 'non-adherent cell population consequent upon feeding the cultures.

Stimulus for Stem Cell Proliferation

We noticed some years ago that the stimulus for stem cell proliferation seemed to be associated with the feeding regime employed [10] Thus, within 24 h following a feed' of the cultures, stem cells present both in the growth medium and in the adherent cell population are in an actively cy-

receptive to the hormone erythropoietin and other molecules necessary for haemoglobinisation to occur [17] Thus far little is known of the regulation of megakaryopoiesis – although potential humoral factors are presently being characterised, and colony stimulation molecules necessary for *in vitro* growth of megakaryocyte precursor cells are being isolated [21 22, 28] Finally the role of antigens, mitogens, growth-promoting activities of macrophages and possible humoral factors such as thymopoietin [15] are being investigated in T and B lymphocyte development.

The list is by no means exhaustive, but does serve to show the range of studies being conducted on these more differentiated cell populations and also underlines the major gap in our knowledge – namely the control of the pluripotent stem cell population. In recent years, we have developed a culture system [4 7] whereby haemopoietic stem cell proliferation and differentiation can be maintained for several months, and which seems to be a suitable model system for studying the regulatory molecules and cellular interactions involved in this earliest compartment.

In this communication I would like to review some of the studies performed with these long-term cultures and the application of the technique to the study of haemopoietic dysplasia, *in vitro* leukaemogenesis and proliferation/differentiation control mechanisms.

Establishing the Cultures

To establish the cultures, the contents of a mouse femur are flushed into culture flasks (Corning, 25 cm^2) containing 10 ml of Fischer s medium (Gibco) and 25% horse serum (Flow Laboratories) supplemented with antibiotics as previously described [4]. No attempt is made to obtain a single cell suspension Many batches of horse serum and all batches of fetal calf serum were found to be deficient in their capacity to maintain *in vitro* haemopoiesis, but can be successfully 'reconstituted' by the addition of hydrocortisone as described by *Greenberger* [16] and confirmed by us [unpublished]

The cultures are maintained in air + 5% CO_2 at a temperature of 33 C [7] and are fed weekly by removal of half the growth medium and addition of an equal volume of fresh medium. Over a 2 to 3-week period, an adherent layer of cells is formed (see later) and the cultures are then re-inoculated with a further population of 5×10^6–10^7 syngeneic or allogeneic marrow cells [6, 7 12] These constitute the cultures proper and are fed weekly by demi-depopulation as described earlier The growth medium removed contains cells in suspension which, after appropriate concentration or dilution, can be assayed for the haemopoietic stem cells (CFU S) [26] or the variety of committed progenitor cells. In this way events occurring in individual cultures can be monitored over many weeks.

The 'Non-Adherent Cells

These cells show an approximate doubling after each 'feed' and consist mainly of granulocytes in all stages of maturation [7 11] Megakaryocytes are also being produced, but the cultures contain no morphologically recognisable erythroid cells or lymphocytes [2, 7] Pluripotent stem cells (CFU-S) are being produced for periods of 10–18

...ks, again showing an approximate dou- between each feed. Such stem cells are quite normal [7] forming all ... elements and protecting mice potentially lethal radiation when in- jected in vivo. Along with stem cell prolif- eration there is a production of CFU-C [7 27] BFU-E [25] and megakaryocyte precursor cells [28]. Recent evidence also indicates the prolonged maintenance of a lymphoid 'stem' cell (restricted to B and T lymphopoiesis) [23] and of a pre T' lymphoid cell population [*Phillips* personal commun., 1978]. Therefore, the cultures *produce* the whole range of differ- entiated progeny for many weeks, although, within the cultures, *further differentiation* and maturation is limited to the granulocyte megakaryocyte lineages.

The Adherent Cells

In the absence of an adherent layer stem cells rapidly decline and disappear within a few days. Conclusive evidence for the role the adherent cell population in the long- cultures comes from our studies with genetically anaemic mutant mice [8]. Two strains are of importance: Sl/Sl^d mice which have a defective inductive environ- ment (but normal stem cells) and W/W^v mice which have an intrinsic defect in the stem cell population (although the inductive environment is quite normal). Using the long-term cultures, we showed that these defects can be reproduced *in vitro* [8] and that they can be overcome using the appro- priate combination of adherent layer and stem cells. For example, adherent layers from Sl/Sl mice (defective) re-inoculated with marrow stem cells from W/W^v mice (defective) showed no maintenance of hae- mopoiesis, whereas the reverse combination (W/W^v re-inoculated with Sl/Sl^d) showed a prolonged *in vitro* haemopoiesis. The conclusion was that stem cell proliferation *in vitro* depends upon interactions between a competent stem cell population and the appropriate inductive environment.

This begs the question as to the cell pop- ulation(s) which make up the adherent lay- er. Careful ultrastructural analysis has shown that at least three cell populations are pres- ent – which are probably representative of marrow stromal elements *in vivo* [1 2, 7 11]. These *in vitro* populations are made up of cells forming a flattened pavement-like structure (which we speculate are derived from the endothelium of the marrow sinus), macrophages and fat cells. The three cell types are often found in intimate association as a complex cellular multilayer [1 2] often enclosing areas of active haemopoiesis.

The nature of the adherent layer suggest- ed to us that the commitment for stem cell proliferation and differentiation arises from cellular interactions occurring in the adher- ent layer. Certainly the adherent layer is a major *site* of stem cell production [5 10] and regularly regenerates the stem cells re- moved from the 'non-adherent' cell popula- tion consequent upon feeding the cultures.

Stimulus for Stem Cell Proliferation

We noticed some years ago that the sti- mulus for stem cell proliferation seemed to be associated with the feeding regime em- ployed [10]. Thus, within 24 h following a 'feed' of the cultures, stem cells present both in the growth medium and in the ad- herent cell population are in an actively cy

receptive to the hormone erythropoietin and other molecules necessary for haemoglobinisation to occur [17] Thus far little is known of the regulation of megakaryopoiesis – although potential humoral factors are presently being characterised, and colony stimulation molecules necessary for *in vitro* growth of megakaryocyte precursor cells are being isolated [21 22, 28] Finally the role of antigens, mitogens, growth-promoting activities of macrophages and possible humoral factors such as thymopoietin [15] are being investigated in T and B lymphocyte development.

The list is by no means exhaustive but does serve to show the range of studies being conducted on these more differentiated cell populations and also underlines the major gap in our knowledge – namely the control of the pluripotent stem cell population In recent years, we have developed a culture system [4 7] whereby haemopoietic stem cell proliferation and differentiation can be maintained for several months, and which seems to be a suitable model system for studying the regulatory molecules and cellular interactions involved in this earliest compartment.

In this communication I would like to review some of the studies performed with these long term cultures and the application of the technique to the study of haemopoietic dysplasia, *in vitro* leukaemogenesis and proliferation/differentiation control mechanisms.

Establishing the Cultures

To establish the cultures, the contents of a mouse femur are flushed into culture flasks (Corning, 25 cm^2) containing 10 ml of Fischer's medium (Gibco) and 25% horse serum (Flow Laboratories) supplemented with antibiotics as previously described [4]. No attempt is made to obtain a single cell suspension. Many batches of horse serum and all batches of fetal calf serum were found to be deficient in their capacity to maintain *in vitro* haemopoiesis, but can be successfully 'reconstituted' by the addition of hydrocortisone as described by *Greenberger* [16] and confirmed by us [unpublished].

The cultures are maintained in air + 5% CO_2 at a temperature of 33 °C [7] and are fed weekly by removal of half the growth medium and addition of an equal volume of fresh medium. Over a 2 to 3-week period, an adherent layer of cells is formed (see later) and the cultures are then re-inoculated with a further population of 5×10^6–10^7 syngeneic or allogeneic marrow cells [6, 7 12] These constitute the cultures proper and are fed weekly by demi-depopulation as described earlier The growth medium removed contains cells in suspension which, after appropriate concentration or dilution, can be assayed for the haemopoietic stem cells (CFU S) [26] or the variety of committed progenitor cells. In this way events occurring in individual cultures can be monitored over many weeks.

The 'Non-Adherent' Cells

These cells show an approximate doubling after each 'feed' and consist mainly of granulocytes in all stages of maturation [7 11] Megakaryocytes are also being produced but the cultures contain no morphologically recognisable erythroid cells or lymphocytes [2, 7] Pluripotent stem cells (CFU-S) are being produced for periods of 10–18

weeks, again showing an approximate doubling between each feed. Such stem cells are functionally quite normal [7] forming all haemopoietic elements and protecting mice from potentially lethal radiation when injected *in vivo* Along with stem cell proliferation there is a production of CFU-C [7 27] BFU-E [25] and megakaryocyte precursor cells [28] Recent evidence also indicates the prolonged maintenance of a committed lymphoid stem cell (restricted to B and T lymphopoiesis) [23] and of a 'pre T' lymphoid cell population [*Phillips* personal commun. 1978] Therefore, the cultures produce the whole range of differentiated progeny for many weeks, although, within the cultures, further differentiation and maturation is limited to the granulocyte and megakaryocyte lineages.

The Adherent Cells

In the absence of an adherent layer stem ...ils rapidly decline and disappear within a ..w days. Conclusive evidence for the role of the adherent cell population in the long-term cultures comes from our studies with ... / anaemic mutant mice [8] Two ... are of importance $S1/S1^{d}$ mice ...hich have a defective inductive environ... (but normal stem cells) and W/W^{v} which have an intrinsic defect in the ...tem cell population (although the inductive ...ent is quite normal). Using the ...ong-term cultures, we showed that these ... can be reproduced *in vitro* [8] and they can be overcome using the appro... combination of adherent layer and ...tem cells. For example adherent layers ...rom S1/S1 mice (defective) re-inoculated ...ith marrow stem cells from W/W^{v} mice (defective) showed no maintenance of haemopoiesis, whereas the reverse combination (W/W^{v} re-inoculated with $S1/S1^{d}$) showed a prolonged *in vitro* haemopoesis. The conclusion was that stem cell proliferation *in vitro* depends upon interactions between a competent stem cell population and the appropriate inductive environment.

This begs the question as to the cell population(s) which make up the adherent layer Careful ultrastructural analysis has shown that at least three cell populations are present – which are probably representative of marrow stromal elements *in vivo* [1 2, 7 11] These *in vitro* populations are made up of cells forming a flattened pavement-like structure (which we speculate are derived from the endothelium of the marrow sinus), macrophages and fat cells. The three cell types are often found in intimate association as a complex cellular multilayer [1, 2] often enclosing areas of active haemopoiesis.

The nature of the adherent layer suggested to us that the commitment for stem cell proliferation and differentiation arises from cellular interactions occurring in the adherent layer Certainly the adherent layer is a major *site* of stem cell production [5 10] and regularly regenerates the stem cells removed from the 'non-adherent' cell population consequent upon feeding the cultures.

Stimulus for Stem Cell Proliferation

We noticed some years ago that the stimulus for stem cell proliferation seemed to be associated with the feeding regime employed [10] Thus, within 24 h following a 'feed' of the cultures stem cells present both in the growth medium and in the adherent cell population are in an actively cy

receptive to the hormone erythropoietin and other molecules necessary for haemoglobin isation to occur [17] Thus far little is known of the regulation of megakaryopoie sis – although potential humoral factors are presently being characterised and colony stimulation molecules necessary for *in vitro* growth of megakaryocyte precursor cells are being isolated [21 22, 28] Finally the role of antigens, mitogens, growth promoting ac tivities of macrophages and possible humoral factors such as thymopoietin [15] are being investigated in T and B lymphocyte development.

The list is by no means exhaustive, but does serve to show the range of studies being conducted on these more differentiat ed cell populations and also underlines the major gap in our knowledge – namely the control of the pluripotent stem cell population In recent years, we have developed a culture system [4 7] whereby haemopoietic stem cell proliferation and differentiation can be maintained for several months, and which seems to be a suitable model system for studying the regulatory molecules and cellular interactions involved in this earliest compartment.

In this communication I would like to re view some of the studies performed with these long-term cultures and the application of the technique to the study of haemopoiet ic dysplasia, *in vitro* leukaemogenesis and proliferation/differentiation control mecha nisms.

Establishing the Cultures

To establish the cultures, the contents of a mouse femur are flushed into culture flasks (Corning, 25 cm^2) containing 10 ml of Fischer s medium (Gibco) and 25% horse serum (Flow Laboratories) supplemented with antibiotics as previously described [4]. No attempt is made to obtain a single cell suspension. Many batches of horse serum and all batches of fetal calf serum were found to be deficient in their capacity to maintain *in vitro* haemopoiesis, but can be successfully reconstituted by the addition of hydrocortisone as described by *Greenberger* [16] and confirmed by us [unpublished].

The cultures are maintained in air + 5% CO_2 at a temperature of 33 C [7] and are fed weekly by removal of half the growth medium and addition of an equal volume of fresh medium. Over a 2 to 3-week period, an adherent layer of cells is formed (see lat er) and the cultures are then re-inoculated with a further population of 5×10^6–10^7 syngeneic or allogeneic marrow cells [6, 7 12] These constitute the cultures proper and are fed weekly by demi-depopulation as described earlier The growth medium removed contains cells in suspension which, after appropriate concentration or dilution, can be assayed for the haemopoietic stem cells (CFU-S) [26] or the variety of committed progenitor cells. In this way events occurring in individual cultures can be monitored over many weeks.

The 'Non-Adherent Cells

These cells show an approximate doubling after each feed' and consist mainly of granulocytes in all stages of maturation [7 11] Megakaryocytes are also being produced, but the cultures contain no morphologically recognisable erythroid cells or lymphocytes [2, 7] Pluripotent stem cells (CFU-S) are being produced for periods of 10–18

cells produced (e.g. a shift to the 'left') and alterations in the functional capacity of the stem cells. The investigation of such 'preleukaemia' states is currently in progress and may give exciting information as to the nature of the target cells in leukaemogenesis, the mechanism(s) of resistance to leukaemic transformation exhibited by certain mouse strains and the possibility of 'reverting' the cells into a normal differentiation pathway.

Bone Marrow Transplantation

Marrow transplantation is being used increasingly for severe combined immunodeficiency disease, severe aplasias and acute leukaemias. A major problem with such transplants is the development of graft-versus-host disease, which is produced by T lymphocytes present in the original marrow inoculated. As discussed previously the long-term cultures are devoid of lymphocytes – raising the possibility of using such cells for transplantation purposes. In a recent report [12] we showed that such cultured cells could indeed fully reconstitute the haemopoietic system of potentially lethally irradiated allogeneic mice, without the concomitant development of graft versus-host disease. Such work certainly raises the possibility of exploiting such culture systems in human marrow transplantations.

Bone Marrow Aplasias

Long-term bone marrow cultures are also being used for the study of the variety of marrow aplasias induced by irradiation or chemicals. The previous comments on Sl/Sl^d and W/W^v cultures – where *in vitro* haemopoiesis was shown to depend upon interactions between an inductively competent adherent layer (acting as a haemopoietic environment) and competent stem cells – indicate the possibility of exploiting such a culture system in investigating whether defective haemopoiesis is determined by intrinsic stem cell defects or damage to the inductive stroma [5]. In one model system (using repeated exposure to sub-acute X-radiation) we have shown that the haemopoietic aplasia arising is the result of a dual defect in the stem cells and in the environment [14]. In more recent work, myleran treatment was shown to induce essentially a stem cell defect [*Dexter and Schofield*, unpublished observations]. Hopefully such culture systems will eventually incorporate the wider analysis of the aetiology of human marrow aplasias.

Conclusions

The rapid growth in our ability to grow haemopoietic stem cells *in vitro* and the development of clonal assay systems for the variety of committed progenitor cells, is allowing studies to be performed on the factors regulating haemopoiesis. Regulatory networks are being exposed and inroads are being made into mechanisms of leukaemic transformation. The possible usefulness of such techniques in the determination of intrinsic stem cell defects versus microenvironmental influences in a variety of haemopoietic aplasias is currently under investigation and consideration is being given to the application of long-term cultures in bone marrow transplantation.

Acknowledgements

This work was supported by the Cancer Research Campaign and the Medical Research Coun-

cling state. This progressively falls over the next 2–3 days so that between 5 and 10 days post re-feeding, the CFU-S are in a non-cycling state. A further feed is then followed by re-stimulation of the CFU-S and so on. Recent evidence from these laboratories suggest that the stem cell cycling is modulated by production of specific stimulators and inhibitors of stem cell proliferation of the type described by *Lord et al* [19 20] This work, as yet unpublished, opens up exciting possibilities for manipulating the proliferative activity of stem cell populations for investigating the effects of leukaemogenic agents and differentiation stimuli.

The Regulation of Granulopoiesis in Long-Term Cultures

Apart from the described studies on stem cell population, we have also been concerned with the factors regulating granulocyte differentiation. The observations that neither CSF activity [7 27] nor CSF inhibitory material [27] can be recovered from the growth medium of the long term cultures raised the intriguing possibility that the production of granulocytes and macrophages may be occurring in the absence of CSF In recent work [*Dexter and Shadduck*, in preparation] we have shown that addition of an antiserum varied against L-cell CSF has no effect upon haemopoiesis in these cultures. Furthermore, addition of highly purified CSF preparations leads neither to a stimulation nor inhibition of granulocyte/macrophage development. These data indicate that, at best, CSF has only a marginal role in the maintenance of *in vitro* haemopoiesis (granulocyte/macrophage production), and also indicate that granulocyte/macrophage production is already occurring at an optimal rate with further stimulation of CFU-C being prevented (even in the presence of excess levels of CSF).

Other Applications of Long-Term Cultures

In vitro *Leukaemogenesis*

Numerous agents (chemical, viral, X rays) are known to induce leukaemia *in vivo* but the events occurring in the period between initial treatment and the development of overt leukaemia are still a mystery However the development of the long-term culture system is allowing us to instigate some of these events – in particular the progression of changes associated with leukaemic transformation. We have shown that following culture of bone marrow cells treated with the chemical leukaemogen methylnitrosourea, an initial normal haemopoiesis is followed by a decline in normal stem cells and an accumulation of primitive blasts. Inoculation of these blast cells *in vivo* regularly produces a transplantable lymphoma possessing some T-cell characteristics. Furthermore, following *in vitro* treatment with a range of leukaemia viruses (Friend LV Abelson LV FBJ osteosarcoma virus), we have been able to induce a whole variety of haemopoietic dysplasia – ranging from the production of (non-leukaemic) stem cells with an abnormal differentiation capacity through chronic myeloid leukaemic transformation, to overt, blast cell (AML) like leukaemias [9 13 24] B cell-type leukaemias can also be induced [23] Of some interest is that the leukaemic transformation is preceded in all cases by an apparently normal haemopoiesis, followed by morphological changes in the

is produced (e.g. a shift to the "left") and ions in the functional capacity of the cells. The investigation of such pre states is currently in progress may give exciting information as to the of the target cells in leukaemogeno- the mechanism(s) of resistance to leu- transformation exhibited by certain strains and the possibility of 'revert the cells into a normal differentiation thway

Bone Marrow Transplantation

Marrow transplantation is being used in- y for severe combined immunode- disease, severe aplasias and acute as. A major problem with such lants is the development of graft ver -host disease, which is produced by T mphocytes present in the original marrow lated. As discussed previously the ong-term cultures are devoid of lympho- – raising the possibility of using such for transplantation purposes. In a re report [12] we showed that such cul- cells could indeed fully reconstitute haemopoietic system of potentially le- ally irradiated allogeneic mice, without he concomitant development of graf-ver us-host disease. Such work certainly raises he possibility of exploiting such culture sys- in human marrow transplantations.

Bone Marrow Aplasias

Long-term bone marrow cultures are being used for the study of the variety of marrow aplasias induced by irradiation or chemicals. The previous comments on Sl/Sl^{d} and W/W^{v} cultures – where *in vitro* haemopoiesis was shown to depend upon interactions between an inductively competent adherent layer (acting as a haemopoietic environment) and competent stem cells – indicate the possibility of exploiting such a culture system in investigating whether defec tive haemopoiesis is determined by intrinsic stem cell defects or damage to the inductive stroma [5] In one model system (using repeated exposure to sub-acute X-radiation) we have shown that the haemopoietic apla sia arising is the result of a dual defect in the stem cells and in the environment [14] In more recent work, myleran treatment was shown to induce essentially a stem cell defect [*Dexter and Schofield* unpublished observations] Hopefully such culture systems will eventually incorporate the wider analysis of the aetiology of human marrow aplasias.

Conclusions

The rapid growth in our ability to grow haemopoietic stem cells *in vitro* and the development of clonal assay systems for the variety of committed progenitor cells, is allowing studies to be performed on the factors regulating haemopoiesis. Regulatory networks are being exposed and inroads are being made into mechanisms of leukaemic transformation. The possible usefulness of such techniques in the determination *of in* trinsic stem cell defects versus microenvironmental influences in a variety of haemopoietic aplasias is currently under investigation and consideration is being given to the application of long-term cultures in bone marrow transplantation.

Acknowledgements

This work was supported by the Cancer Re search Campaign and the Medical Research Coun-

cil. The author is a Fellow of the Cancer Research Campaign.

References

1 Allen, T D and Dexter T M. Cellular inter relationships during *in vitro* granulopoiesis. Differentiation 6, 191–194 (1976)

2 Allen, T D Stem cells and tissue homoeostasis, pp. 217–240 (*Cambridge University Press*, Cambridge 1978)

3 Broxmeyer H. E., Smithyman, A. Eger R. R., Meyers, P A., and Sousa, M. de Identification of lactoferrin as the granulocyte-derived inhibitor of colony-stimulating activity production. J exp Med. *145* 1052–1067 (1978).

4 Dexter T M and Testa, N G Methods in cell biology vol. 14 pp 387–405 (Academic Press, New York 1976)

5 Dexter T M., Allen, T D and Lajtha, L G in Molecular control of proliferation and differentiation, pp 149–160 (Academic Press, New York 1977).

6 Dexter T M Moore, M. A. S., and Sheridan, A. P C. *Maintenance of hemopoietic stem* cells and production of differentiated progeny in allogeneic and semi-allogeneic bone marrow chimeras *in vitro* J exp. Med. *145* 1612–1616 (1977).

7 Dexter T M., Allen, T D and Lajtha, L. G Conditions controlling the proliferation of haemopoietic stem cells *in vitro*. J cell. Physiol. *91* 335–344 (1977)

8 Dexter T. M. and Moore, M. A. S. *In vitro* duplication and cure of haemopoietic defects in genetically anaemic mice. Nature, Lond. *269* 412–414 (1977).

9 Dexter T M Scott, D and Teich, N M. Infection of bone marrow cells *in vitro* with FLV effects on stem cell proliferation, differentiation and leukaemogenic capacity Cell *12* 355–364 (1977)

10 Dexter T M., Wright, E. G., Krizsa, F and Lajtha, L. G *Regulation of haemopoietic* stem cell proliferation in long-term marrow cultures. Biomedicine 2? 344–349 (1977).

11 Dexter T M Allen, T D Lajtha, L. G., Krizsa, F., Testa, N G and Moore, M. A. S. in Differentiation of normal and neoplastic haematopoietic cells, pp. 63–80 (Cold Spring Harbor Laboratory New York 1978).

12 Dexter T M. and Spooncer E. Loss of immunoreactivity in long-term bone marrow culture. Nature, Lond. *275* 135–136 (1978).

13 Dexter T M., Allen, T D Scott, D and Teich, N M. Isolation and characterisation of a bipotential haemopoietic cell line. Nature, Lond. *277* 471–474 (1979).

14 Dexter T M., Schofield, R., Hendry J and Testa, N G Aplastic Anemia. Pathophysiology and approaches to therapy (Springer Berlin, in press).

15 Goldstein, G In Differentiation of normal and neoplastic hematopoietic cells, pp. 455–465 (Cold Spring Harbor Laboratory New York 1978).

16 Greenberger J S., Sensitivity of corticosteroid-dependent insulin-resistant lipogenesis in marrow pre-adipocytes of obese-diabetic (db/db) mice. Nature, Lond. *275* 752 754 (1978).

17 Iscove, N N. In Hematopoietic cell differentiation, pp 37–52 (Academic Press, New York 1978).

18 Kurland, J I., Bockman, R., Broxmeyer H. E., and Moore, M A. S. *Limitation* of excessive myelopoiesis by the intrinsic modulation of macrophage-derived prostaglandin E. Science *199* 552–555 (1978).

19 Lord, B I., Mori, K. J Wright, G G and Lajtha, L. G A stimulator of stem cell proliferation in regenerating bone marrow Biomed. Express *27* 223–226 (1977).

20 Lord, B I., Mori, K. J Wright, G G and Lajtha, L. G An inhibition of stem cell proliferation in normal bone marrow *Br J Haemat. 34* 441–445 (1976)

21 McDonald, T P *Thrombopoietin production* by human embryonic kidney cells in culture. J Lab. *clin. Med. 85 59 (1975).*

22 Metcalf, D Hemopoietic colonies. *In vitro* cloning of normal and leukemic *cells (Springer* Berlin 1977).

23 Phillips, R. A., Jones, E. V and Miller R. G In Hematopoietic cell differentiation, pp. 129–139 (Academic Press, New York 1978).

24 Teich, N. M and Dexter T M., *In Differentiation* of normal and neoplastic hematopoietic cells, pp. 657–670 (*Cold Spring Harbor* Laboratory New York 1978)

Testa, N. G. and Dexter, T. M.: Long term production of erythroid precursor cells (BFU) in bone marrow cultures. Differentiation 9: 193–195 (1977).
Till, J. E., and McCulloch, E. A.: A direct measurement of the radiation sensitivity of normal mouse bone marrow cells. Radiat. Res. 14: 213–222 (1961).
27 Williams, W., Jackson, H., and Rabellino, E. M.: Proliferation and differentiation of normal granulopoietic cells in continuous bone marrow cultures. J. cell. Physiol. 93: 435–440 (1977).
28 Williams, N., Jackson, H., Sheridan, A. P. C., Murphy, M. J., Elste, A., and Moore, M. A. S.: Regulation of megakaryopoiesis in long-term murine bone marrow cultures. Blood 51: 245–255 (1978).

T. M. Dexter, Paterson Laboratories,
Christie Hospital and Holt Radium Institute,
Manchester M20 9Bx (England)

cll The author is a Fellow of the Cancer Research Campaign.

References

1 Allen, T D and Dexter T M Cellular inter relationships during *in vitro* granulopoiesis. Differentiation 6. 191–194 (1976)

2 Allen, T D Stem cells and *tissue homeostasis*, pp 217–40 (Cambridge University Press, Cambridge 1978)

3 Broxmeyer H. E. Smithyman, A. Eger R. R., Meyers, P A., and Sousa, M. de Identification *of lactoferrin* as the granulocyte-derived inhibitor of colony-stimulating activity production. J exp. Med. *145* 1052–1067 (1978)

4 Dexter T M and Testa, N G *Methods in* cell biology vol 14 pp 387–405 (Academic Press, New York 1976).

5 Dexter T M., Allen, T D and Lajtha, L G in Molecular control of proliferation and dif *ferentiation*, pp. *149–160* (*Academic* Press, New York 1977)

6 Dexter T M., Moore, M. A. S., and Sheridan, A. P C. Maintenance of hemopoietic stem cells and production of differentiated progeny *in allogeneic and semi-allogeneic bone marrow* chimeras *in vitro* J exp Med. *145* 1612–1616 (1977).

7 Dexter T M., Allen, T D and Lajtha, L G Conditions controlling the proliferation of hae*mopoietic stem cells in vitro J cell Physiol. 91* 335–344 (1977)

8 Dexter T M. and Moore, M. A. S. *In vitro* duplication and 'cure of haemopoietic defects in genetically anaemic mice. Nature, Lond. *269 412–414 (1977).*

9 Dexter T M. Scott, D and Teich, N M Infection of bone marrow cells *in vitro* with FLV effects on stem cell proliferation, differ entiation and leukaemogenic capacity Cell *12* 355–364 (1977)

10 Dexter T M., Wright, E. G., Krizsa, F and Lajtha, L G Regulation *of haemopoietic* stem cell proliferation in long-term marrow cultures. Biomedicine 27 344–349 (1977).

11 Dexter T M Allen, *T D* Lajtha, L G., Krizsa, F Testa, N G and Moore, M. A. S. in Differentiation *of normal* and neoplastic hematopoietic cells, pp *63–80* (*Cold Spring Harbor* Laboratory New York 1978).

12 Dexter T M. and Spooncer E. Loss of immunoreactivity in long-term bone marrow cul ture. Nature, Lond. *275* 135–136 (1978).

13 Dexter T M., Allen, T D., *Scott, D and* Teich, N M., Isolation and characterisation of a bipotential haemopoietic cell line. Nature, Lond. 277 471–474 (1979)

14 Dexter T M., Schofield, R., Hendry J and *Testa, N G* Aplastic Anemia. Pathophysiology and approaches to therapy (Springer Berlin, in press)

15 Goldstein, G in Differentiation of normal and neoplastic hematopoietic cells, pp. 455–465 (Cold Spring Harbor Laboratory New York 1978)

16 Greenberger J S., Sensitivity of corticoster *oid-dependent insulin-resistant lipogenesis* in marrow pre-adipocytes of obese-diabetic (db/db) mice. Nature, Lond. *275 752 754* (1978)

17 Iscove, N N in Hematopoietic cell differentiation, pp. *37–52* (*Academic Press, New York* 1978)

18 Kurland, J I. Bockman, R., Broxmeyer *H* E., and Moore, M. A. S. Limitation of excessive myelopoiesis by the intrinsic modulation of *macrophage-derived prostaglandin* E. Science *199* 552–555 (1978).

19 Lord, B I., Mori, K. J Wright, *G G and* Lajtha, L G A stimulator of stem cell prolif eration in regenerating bone marrow Biomed. Express *27 223–226 (1977).*

20 Lord, B I., Mori, K. J., Wright, G. G and Lajtha, L G An inhibition of stem *cell prolif* eration in normal bone marrow Br J Haemat. *34* 441–445 (1976).

21 McDonald, T P Thrombopoietin production by human embryonic kidney cells in culture. *J* Lab. clin. Med. *85 59 (1975).*

22 Metcalf, D Hemopoietic colonies. *In vitro* cloning of normal and *leukemic cells (Springer Berlin 1977)*

23 Phillips, R. A. Jones, E. V and Miller R. G *in Hematopoietic* cell differentiation, pp. *129–* 139 (Academic Press, New York 1978)

24 Teich, N M and *Dexter T M in Differen*tiation of normal and neoplastic hematopoietic cells, pp. 657–670 (Cold Spring Harbor Laboratory New York 1978).

into morphologically recognizable myeloblasts, divide to give promyelocytes and promonocytes until after further division and maturation the mature cells leave the bone marrow

The colony forming unit culture (CFU-C) seems to be a heterogeneous population of cells showing different sedimentation rates in density gradients [29] As a consequence there exists great heterogeneity in size and shape of granulocyte/macrophage colonies in the agar culture [7 9 17] However the heterogeneity in CFU-C and of the mature cells does not necessarily indicate that functionally different CSA are involved. On the contrary recent evidence suggests that different thresholds of responsiveness may be involved. Thus, high concentrations of CSA tend to stimulate granulocyte colony formation, whereas low concentrations stimulate macrophage colony formation.

Colony stimulating activity is required for each cell division and can influence RNA synthesis rates and probably certain functional activities in mature cells. This supposed dual action of CSA would therefore be similar to that of erythropoetin in stimulating erythropoiesis and hemoglobin synthesis as well. Our current knowledge on the regulation of neutrophil and macrophage production by CSA almost entirely originates from cultures of progenitor cells in semisolid agar or methylcellulose [4 12, 14 0]

At present there exists no final proof that CSA stimulates granulopoiesis *in vivo*, although indirect results from several studies appear to make it a good candidate. Thus, diffusion chamber methods in mice murine models of inflammatory exudates and transplantation experiments with CSA secreting tumors strongly suggest an *in vivo* action of CSA [3 13]

Despite a number of studies in the past 10 years it is still not clear which cell in the body produces CSA, since it can be extracted from supernatants of almost all tissues. There exist two alternatives (1) CSA can potentially be produced by most cells in the body or (2) certain cells common to all tissues, like macrophages or lymphocytes, are the source of CSA. The ability of certain cell lines such as L cells [26] or melanoma cells to produce CSA cannot contribute to this problem because these cells may have an abnormally depressed genome.

CSA stimulating human CFU-C was detected only in human and in some monkey sera [17] and in conditioned medium of human tissues, which suggests a certain species specificity of CSA [5 8, 22, 27]

We started characterizing human CSA and have chosen, because of their easy availability human leukocytes to be the source of CSA. Attempts to characterize CSA from human sources have shown a great heterogeneity in molecular weight. Leukocyte-conditioned media contained four molecular species with 1,000, 15,000, 35,000 and 90,000 daltons, respectively [21 23 27] Mononuclear leukocytes produced two CSA, both active on mouse and human CFU-C with 25,000 and 35,000 molecular weight according to their origin. In addition, several activities have been described in lung-conditioned medium as well as in placenta-conditioned medium [5 7]

Inhibition of CFU-C by Chalones As in other tissues, a cell line-specific but not species-specific inhibitor called chalone has been described [18] Its inhibitory activity has been shown in the culture system [15 16] and perhaps *in vivo* [24] and it was postulated that it might be a granulocyte product. The subcellular origin of this granulo-

Acta haemat. 62. 306-314 (1979)

Humoral Factors Modulating Growth of Granulocyte Macrophage Progenitor Cells

K. Havemann W D Gassel and H Laukel

Medizinische Klinik und Medizinische Poliklinik, Marburg

Key Words. Colony forming cell Colony-stimulating activity Fibrinogen degradation products Peripheral leukocytes Spleen cells

Abstract. *The kinetic of production of colony-stimulating activity (CSA) inducing mouse* and *human* colony-forming cells (CFU-C) was tested in different *human leukocyte culture* systems. Stimulated and unstimulated *cultures of* spleen single cell suspensions, peripheral mononuclear leukocytes and acute monocytic leukemia (AMoL) cells were investigated. With the exception of the AMoL cells, stimulated *cultures* always revealed higher CSA levels than unstimulated controls. The spleen cell cultures *exhibited the highest* overall activity showing three molecular *species of 70 000 35 000* and *10 000* daltons activating human CFU-C to *form colonies in the* agar culture system. Furthermore it could be demonstrated that colony *formation* could be inhibited by low molecular weight fibrinogen degradation *products obtained by digestion of fibrinogen with* granulocyte-derived elastase.

Introduction

The hemopoietic system is *comprised* on three main compartments. the pluripotential stem cells (CFU S) the committed stem cells (CFU-C, CFU E, etc.) and the differentiated compartment with its morphologically identifiable cells. The pluripotential stem cell has the capability of self renewal and control of the compartment size. Regulation of these cells is still a *matter of* discussion. Short range regulatory interactions with the microenvironment as well as long-range humoral factors have been described, but exact knowledge is lacking

Our knowledge on the regulation of CFU-C, *the committed granulocyte/ macrophage progenitor cells*, however is more precise. *These cells normally originate* from pluripotential cells *going into cell cycle* [2] Once the cell has become a CFU-C, the amplification and differentiation is controlled by humoral factors which are highly specific. Upon stimulation by these factors, given the operational term colony-stimulating activity (CSA) the CFU-C differentiate

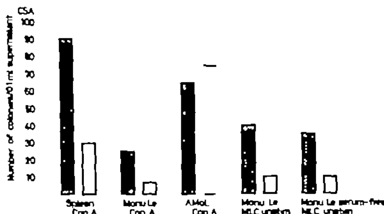


Fig. 2. Production of CSA by leukocyte cultures. The activity of stimulated (stripped columns) and unstimulated cultures (open columns) is shown. From left to right: spleen cells, peripheral mononuclear leukocytes, peripheral leukocytes of acute monocytic leukemia all Concanavalin A-stimulated, MLC stimulated peripheral mononuclear leukocyte cultures with and without serum.

While unstimulated cells only released small amounts of CSA, cultures incubated with 5 μg Con A/ml for 48 h gave the best yield. We furthermore compared mitogen and allogeneic cell-stimulated peripheral mononuclear cells, mitogen-stimulated spleen cells and cells from an acute monocytic leukemia (AMoL) for their production of CSA (fig. 2). With the exception of the leukemic cells, which already showed high spontaneous production without further augmentation after stimulation, all stimulated cultures revealed a marked increase of CSA production as compared to unstimulated controls. Stimulated spleen cell cultures showed a significant higher yield than comparable numbers of mononuclear cells. Since the number of spleen cells obtained from one spleen was about 30 times higher than that of mononuclear cells from one blood pack, the total amount of CSA produced by one spleen was approximately 100-fold with about 5×10^6 overall induced colonies. Therefore the spleen cell cultures have been used for most of the following experiments.

Table I. Combination of different CSA species in different leukocyte supernatants and their isoelectric points (IEP)

Material	70,000 daltons	35,000 daltons	10,000 daltons
MNC + Con A	+	+	+
MLC	−	+	+
Spleen + Con A	−	+	+
AMoL	−	+	+
IEP	7.0 7.7	4.2 5.0	5.6 6.4

MNC = Peripheral mononuclear cells, MLC = mixed lymphocyte culture, Spleen = single cell suspension of homogenized spleen, AMoL = acute monocytic leukemia, + Con A = Concanavalin A-stimulated

When testing the molecular weight distribution of human activities from different leukocyte sources, up to three molecular

cyte product is unknown. We here present first evidence that it might be an interaction product of neutral granulocyte protease with a coagulation factor

Material and Methods

Mouse bone marrow cultures were performed in 0.3% agar according to *Bradley and Metcalf* [4]. For human cultures bone marrow was obtained by diagnostic puncture of the iliac crest in patients with nonhematological diseases and anemia. Cultures were set up following the procedure of *Pike and Robinson* [19] with 200,000 cells/ml. Cells were preseparated by adherence to Petri dishes in the presence of 50% fetal calf serum and three washings with serum-free medium afterwards.

Lymphocyte cultures were done in the standard manner [9]. Whole blood was separated by Ficoll density gradient centrifugation and either all mononuclear cells or the nonadherent cell fraction were used. Concanavalin A was used as mitogen throughout all experiments in the doses given below.

Human spleens were homogenized with an Ultra Turrax (type 18/10). Afterwards bigger tissue fragments were allowed to sediment and the supernatant (single cell suspension) was used for culture.

Gel chromatography of the conditioned media was performed by Sephadex G 100 chromatography (column 2.6 × 100 cm). For isoelectric focusing we used the LKB apparatus (LKB 2103, 10 W, 200 mA) and the standard Ampholine gel pH 3.5–9.5

Results

A kinetic of CSA production in cultures of spleen cells is given in figure 1. Using a constant cell concentration of 1×10^7 cells, unstimulated cultures and cultures stimulated with increasing doses of Concanavalin A (Con A) followed in parallel.

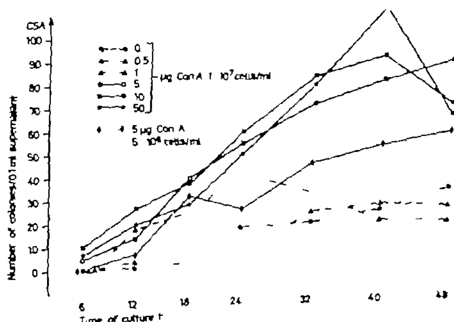


Fig. 1. Kinetic of the production of CSA in spleen cell culture supernatants. 1×10^7 spleen cells were incubated with different doses of concanavalin A and the supernatants harvested at intervals from 6 to 48 h. 5×10^4 cells and 5 µg Con A served as control.

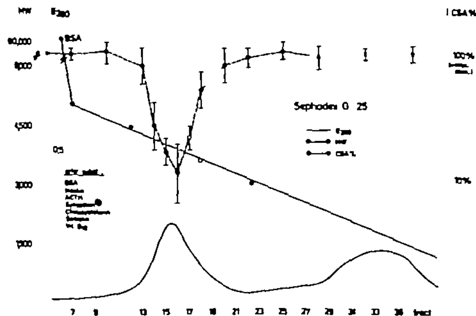


Fig. 4. Sephadex G-25 chromatography of granulocyte extract showing inhibitory material with an approximat molecular weight of 4,000

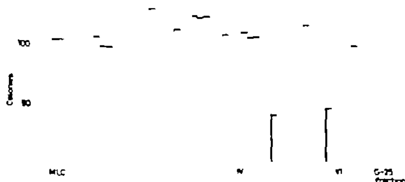


Fig. 5. Inhibition of colony formation by low molecular fibrinogen split products produced in vitro by incubation of fibrinogen with neutral granulocyte protease. Fraction I VI are different pools according to the elution profile of Sephadex G-25 chromatography the molecular weight of pool V and VI is less than 1,000 daltons. All cultures were stimulated by a MLC supernatant (control = MLC, broad column, left). Protein concentration in the different pools undiluted material 13 µg/0.1 ml, dilutions 1:1 1:2, 1:4, 1:8, 1:16, 1:32 in each pool (columns from left to right).

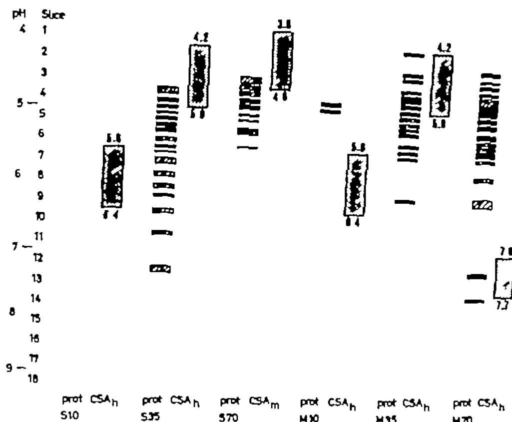


Fig. 3 Results of isoelectric focusing of different CSAs. The stainable protein bands (left, prot.) and the range of eluted activity (right, stripped column) is given for each activity. S = Spleen, M = peripheral mononuclear cells, 10, 35, 70 = daltons × 1 000, CSA_h = CSA stimulating human marrow, CSA_m = CSA stimulating mouse marrow

weight species were detected, i.e. two in spleen cultures, up to three in mitogen or allogeneic cell-stimulated peripheral mononuclear cell cultures and two in cultures of AMoL cells (table I). Although number and concentration of these human activities varied in the different supernatants, they always eluted in Sephadex G-100 chromatography at 70 000, 35 000 and 10 000 daltons. All these molecular species only were active on human CFU-C. In addition, isoelectric focusing of these preseparated activities revealed coincidental isoelectric points of the CSAs with corresponding molecular weights suggesting identity between these activities from different leukocyte sources (fig. 3).

Inhibition of Colony Formation

As PMN (polymorphnuclear leukocytes) are known to inhibit colony formation, we tested granulocyte extracts with respect to their inhibitory capacity. Buffy coat leukocytes treated by hypotonic shock and Ficoll density separation were subjected to sonification. This material was filtrated by Amicon UM 10 filter and the effluent concentrated by a further UM 2 filtration step. Consecutive Sephadex G 25 chromatography revealed a sharp peak of inhibitory activity with an apparent molecular weight of about 4 000 (fig. 4).

Human granulocytic neutral proteases of lysosomal origin are able to degrade differ-

alone would explain most of the phenomena seen, evidence from other studies indicates a complex system of a positive and neg- e feedback control. For instance, while withdrawal from the blood ads to accelerated release of polymor lear granulocytes into circulation, infusion of granulocytes reduces cell release from the bone marrow and inhibition of granulocyte production.

Therefore an additional negative feed- k under the control of inhibitory agents chalones has been suggested. Low molec ar PMN-derived inhibitors have been de [1 15 16 24] which, cell line-specific but not species-specific, inhibit cell ation in the granulocyte/macrophage stem in a reversible manner. This so-called granulocyte chalone is a poly- ide with a molecular weight of about ,000 daltons, which most likely arrests the CFU-C compartment in the G phase. Similar results have been obtained by us. When the ultrafiltrates of granulocyte extracts were separated on gel chromatography a ked inhibition of colony growth was obtained with a peptide of a similar molecular weight. Further experiments with fibrinogen degradation products produced by *in vitro* ion with granulocytic neutral proteases revealed similar inhibitory activity. Although the specificity of this inhibitor for the granulocyte macrophage system has still be proven, these findings might contribute to the mechanism of production of locyte chalones.

Apart from the importance to character humoral stimulators and inh bitors of granulocyte macrophage production, the interest in these regulators has become sharp- increased by the observation that leukemic retain responsiveness to these regulators. This encouraging observation might open the possible use of these humoral regulators in the control of leukemia proliferation.

References

1 Aardale, N P Laerum, O D Pa kowits, D R., and Maurer, H R. Inhibition of agar colony formation by partially purified granulocyte extracts (chalone) Virchows Arch Abt B Zellpath. 24 41 (1977).

2 Baum, S. J Negative and positive feedback control of the committed granulocytic stem cell compartment; in Baum and Ledney E perimental hematology today pp. 177 134 Springer New York 1976)

3 Beran, M Serum levels of colony-stimulating factor( ) and the growth of bone marrow cell in diffusion chambers. Cell Tiss. Kinet. 8 561 568 (1975)

4 Bradley T R and Metcalf, D The growth of mouse bone marrow cells in ro Aust J exp Biol med Sci 44 287 300 (1966)

5 Burgess, A W Wilson, E M A., and Metcalf, D Stimulation by human placental-conditioned medium of hemopoietic colony formation by human bone marrow cells. Blood 49 573–583 (1977)

6 Egbring, R Schmidt, W Fuchs, G and Havemann, K. Demonstration of granulocytic proteases in plasma of patients with acute leukemia and septicemia with coagulation defects Blood 49 19–31 (1977)

7 Engh, G van den, Mokler D Williams, N and Bol, S. Physical characterization of subpopulation of granulocyte monocyte progenitor cells (CFU-C) in Baum and Ledney Experimental hematology today pp 157–164 (Springer New York 1976)

8 Fojo, S S., Wu, M C Gross, M A Purcell, Y and Yunis, A A Purification and characterization of colony-stimulating factor from human lung Biochemistry 17 3109–3115 (1978)

9 Gassel, W -D Ha emann, K., Manteuffel, G F von, and Lenkel, H Colony-stimulating factor (CSF) in human mixed lymphocyte cultures

ent proteins [23] The main enzyme granulocytic elastase, actively secreted under certain conditions rapidly degrades fibrin and fibrinogen in the surrounding environment [10 25] including the fibrinogen sticking to the granulocyte membrane As a consequence, high and low molecular weight fibrinogen split products are formed [6] We tested these split products in the bone marrow agar culture technique While large- and medium-size split products had no effect on the granulocyte macrophage colony formation a peptide with an approximate molecular weight of less than 1 000 daltons strongly inhibited CFU-C proliferation (fig. 5)

Discussion

The aim of the study was to investigate the following questions (1) Which are the best sources of human leukocytes for production of CSA? (2) Does there exist a similarity in molecular weight and isoelectric point among CSAs from different leukocyte sources? (3) Which method can be used for purification of human CSA?

To come up with a sufficient amount of cells for factor production we opened up a new source, namely human spleen cells obtained from patients after splenectomy Using this method, an approximately 100-fold higher yield of CSA is obtained from one spleen as compared to one blood pack. Such a homogeneous material is appropriate for further purification steps and for the characterization of the CSA active on human CFU-C.

Already by gel chromatography and isoelectric focusing the CSA with a molecular weight of 10 000 daltons could be liberated from other protein impurities, and antiserum production began. To further purify the second common factor of the leukocyte supernatants with a molecular weight of 35 000 we developed a combination preparative steps such as gel chromatography anion exchange chromatography C A Sepharose chromatography and hydrophobic interaction chromatography which hopefully will allow purification of this important regulator of hemopoiesis.

The results of the molecular sieve chromatography indicate a great size heterogeneity of functionally equal molecules, a finding which is compatible with the results in a number of lymphokines [28] Since isoelectric points of the different molecular species are rather different, it is unlikely that the heterogeneity is due to multiple forms of a common molecule. Leukopoietins as well as erythropoietins are supposed to behave similarly to hormonal substances, and indeed the molecular heterogeneity of CSA is more comparable to that seen with certain peptide hormones where by a sequence of enzymatic events active peptide hormones of small molecular weight are formed from the ancestral pre and prohormones [11] Our present inability to detect activities like ACTH, growth hormone or the alpha-chain of human chorion gonadotropin in the active supernatants does not necessarily disprove this assumption.

The nature of the CSA-producing cell, which could be the monocyte, the lymphocyte or both, is still unknown However since two of the molecular entities were present in the monocytic leukemia cultures, which almost entirely consisted of abnormal monocytic cells, it is possible that the human activities originate mainly from the monocyte

Although the theory of control of granulocyte/macrophage progenitor cells by CSA

Acta haemat. 62, 315–321 (1979)

Colony Growth of T Lymphocytes *in vitro*

Control and Regulation of Clonal Formation

L. A. Rozenszajn, I. Goldman and A. Zeevi[1]

Department of Life Sciences, Bar-Ilan University, Ramat-G n, and
Department of Medical Laboratories, Meir Hospital, Kfar Saba

Key Words. Lymphokines · Macrophages · Regulation of lymphopoiesis · lymphocyte colony

Abstract. Human lymphocytes stimulated with PHA in liquid phase (step 1) and then d in a two-layer soft agar system (step 2) grew and developed into T cell colonies. ony formation was enhanced when the agar culture was supplemented with culture fluid tained from phytohemagglutinin-treated lymphocytes (Ly-CF). Untreated lymphocytes d directly in the soft agar system also developed into colonies if the culture medium ed Ly-CF. Mitogen-sensitized T lymphocytes produced a lymphocyte colony enhanc factor in the culture fluid which stimulated lymphocytes into colony formation. The best ting efficiency (1:250) was attained when blood mononuclear cells were seeded. When cell culture fluid or a highly concentrated blood-adherent cell population was added the soft agar, colony development was strongly inhibited. Monocytes-macrophages a lymphocyte colony inhibiting factor in the culture medium. These lymphokines a competitive influence on T cells and thus control and regulate clonal proliferation.

Introduction

The hemopoietic system functioning in normal physiologic capacity provides a supply of mature cells. The cell on of the hemopoietic system in-des pluripotent, committed and maturing ls. The pluripotent cells give rise to com-tted stem cells which, after being physio-ically triggered by humoral regulatory s, proliferate, differentiate and mature into the morphologically recognizable cells of each hemopoietic cell lineage. The use of recently developed *in vitro* techniques and assays for growing different classes of hem-opoietic precursor cells led to the accumula-tion of much information and permitted in-sight into the mechanism and control of hemopoiesis and cellular kinetics.

Granulocyte precursor cells (CFU-C) were induced to form colonies of granulo-cytes and macrophages in soft agar in the presence of a colony-stimulating factor [1 1]. Under appropriate culture conditions,

[1] thank Ms. H. Deshen for her editorial tance.

effect on human and mouse progenitor cells. Exp Hematol. 6 391–397 (1978)

10 Gramse, M Bingenheimer C., Schmidt, W., Egbring, R., and Havemann, K. Degradation products of fibrinogen by elastase-like protease from human granulocytes. J clin. Invest. *21* 1027–1033 (1977)

11 Habener J F and Potts, J T., Biosynthesis of parathyroid hormone. New Engl J Med. *299* 635–644 (1978)

12 Iscove, N N Senn, J S. Till, J E., and McCulloch, E. A Colony formation by normal and leukemic marrow cells in culture: effect of conditioned medium of human leukocytes. Blood *37* 1–5 (1971)

13 Keeb G and Lala, P K. Effect of Ehrlich ascites tumor transplantation in mice on the distribution of cells capable of forming hemopoietic colonies *in vitro* Eur J Cancer *14* 331–342 (1978)

14 Laukel, H Gassel, W D Dosch, H M Schmidt, W and Havemann, K. Preparation of colony-stimulating activity from large batches of human urine and production of antisera against it. J cell. Physiol. *94* 21–30 (1978)

15 Maschler R. and Maurer H. R.. Screening for granulopoiesis inhibitors (chalones) by different assays. Hoppe-Seyler's Z. physiol. Chem. *359* 825–834 (1978)

16 Maurer H R., Henry R., and Maschler R. Chalone inhibition of granulocyte colony growth in agar kinetic quantitation by capillary tube scanning. Cell Tiss. Kinet. *11* 129–138 (1978)

17 Moore, M A. S.. *In vitro* studies in the myeloid leukaemias; in Cleton, Crowther and Malpas, Advances in acute leukaemia, pp. 161–228 (North-Holland, Amsterdam 1974).

18 Paukovits, W R. Hinterberger W and Paukovits, J B The granulocyte chalone – a specific inhibitor of granulopoiesis. molecular weight and chemical nature. Oncology *34* 187–189 (1977)

19 Pike, B L. and Robinson, W A.. Human bone marrow colony growth in agar gel. J cell. Physiol *76*. 77–84 (1970).

20 Pluznik, D H. and Sachs, L. The cloning of normal Mast' cells in tissue culture J cell. Physiol *66*. 319–324 (1965).

21 Price, G B., McCulloch, E. A., and Till, J A new low molecular weight granulocyte colony-stimulating activity Blood *42* 341 (1973)

22 Price, G B McCulloch, E A., and Till, J F Cell membranes as sources of granulocyte colony-stimulating activities. Exp. Hematol. 227–233 (1975)

23 Price, G B., Senn, J S., McCulloch, E. A and Till, J E.. The isolation and properties granulocytic colony stimulating activities medium conditioned by human peripheral leukocytes. Biochem. J *148* 209–217 (1975).

24 Rytömaa, T Vilpo, J A., Levanto, A., and Jones, W A.. Effect of granulocytic chalone on acute myeloid leukaemia in man. Lancet i: 771–774 (1977).

25 Schmidt, W., Egbring, R., Havemann, K. and Schmidt, M.. Charakterisierung und biologische Effekte von neutralen Proteasen, in Stacher und Höcker Erkrankungen der Myelopoese, pp 123–136 (Urban & Schwarzenberg, München 1976)

26 Shadduck, R. K. and Metcalf D Preparation and neutralization characteristics of an anti-CSF antibody J cell. Physiol. *86*. 247–252 (1975).

27 Shah, R. G Caporale, L. H., and Moore, M. A. S.. Characterization of colony-stimulating activity produced by human monocytes and phytohemagglutinin-stimulated lymphocytes. Blood *50* 811–821 (1977).

28 Sorg, C. and Geczy C. L.. Antibodies to guinea pig lymphokines. III. Reactions with radiolabeled lymphocyte activation products Eur J Immunol *6*. 688–693 (1976).

29 Visser J., Engh, G van den, Williams, N and Mulder D.. Physical separation of the cycling and noncycling compartment of murine hemopoietic stem cells; in Baum and Ledney Experimental hematology today pp. 1 28 (Springer New York 1976).

K. Havemann,
Medizinische Klinik und
Medizinische Poliklinik,
Emil-Mannkopff Str 1 3550 Marburg (FRG)

Depletion of Phagocytic Cells

Mononuclear cell suspensions were depleted of ytes by the carbonyl iron technique [7] he mononuclear cells (15×10^6) were incubated 1 h at 37 °C in 2 ml EM containing 10%/ HS 20-30 mg carbonyl iron; the suspension was persed by shaking every 15 min. The non-iron-staining cells were separated magnetically from that had ingested iron, washed 3 times with d and the viable cells counted.

Preparation f Ly-CF M-CF and Sp-CF

Blood lymphocytes (1.5×10^6 cells/ml) were tured for 3 days in the presence of PHA-M 0125 ml/ml medium) and 10% HS. The suspen- was centrifuged, the supernatant retained and red at 20 °C [23, 28]. Blood mononuclear (4×10^6/ml) or spleen cells (10^7/ml) that adhered to plastic dishes were cultured in The cell-free medium collected after an incu- ion period of 4 days was used

Soft Agar Culture

T culture methods were employed

T o-Step Method. The isolated cells were sen- zed with PHA (0.0125 ml/ml) in liquid cul- phase for 18 h at 37 °C in 5-7 %/ CO_2 in air subsequently seeded in two-layer soft-agar ture system containing PHA [16]. Liquid cul of phagocyte-depleted lymphocytes or from cell population reconstituted with adherent s to concentrations of 3, 5 10, 15 or 30%/ were When Ly-CF M-CF or Sp-CF were used in culture system, the EM of the lower agar layer replaced by the specific culture fluid. The cul- dishes were incubated at 37 °C in fully fied atmosphere containing 5-7 %/ CO_2. In Colonies were observed and scored under an microscope after 5-6 days of culture.

One-Step Method. Cells not subjected to treat- with PHA were plated directly in the agar ture system to hich Ly-CF had, or had not, added. Colonies were examined and counted 6-7 days of culture. Colonies containing than 50 cells were scored discrete groups of 50 cells were tabulated as clusters

Morphologic Studies

he large colonies growing within the upper layer ere aspirated with capillary tubes, erred to slides, mixed with inactivated HS and dried. The small flat colonies that formed on the surface of the upper layer were harvested by adding 0.5 ml EM to the culture plate, removing them with Pasteur pipet and cytocentrifuging them onto slides. The slides were stained with M y-Grünwald-Giemsa and the morphology of the cells examined.

Lymphoid C ll Surface Markers

Pooled colonies were suspended in EM the cells were tested for E-rosette-forming capacity [27] and for immunoglobulin-bearing cell content [26].

Results

Two-Step Culture Method

When human mitogen-sensitized lymphocytes were cultured in a two-layer soft agar culture system in the presence of PHA, they proliferated and developed into colonies. Two distinct types of colonies de veloped in the upper agar layer: type I, large colonies of 50–500 or more cells which appeared within the upper layer 3–4 days after the cells were seeded, and type II small flat colonies composed of 50–200 cells which formed on the surface of the upper layer after 6–7 days of culture (fig. 1 2).

When the lower agar layer was supple mented with Ly-CF the cloning potential of PHA-treated lymphocytes was enhanced by 70–100% (table I, fig. 3).

If human blood mononuclear cells were seeded instead of isolated lymphocytes, the cloning efficiency improved by more than 100% . Adding Ly-CF to the soft-agar cul ture of seeded mononuclear cells augmented the number of colonies by 200% (table I). The highest cloning efficiency (1:250) was obtained when blood mononuclear cells were cultured in the presence of Ly-CF

When the agar culture system was supplemented with M-CF or Sp-CF an inhibit

colonies containing human eosinophils were observed adjacent to monocyte macrophage colonies [19] Progenitor cells (CFU M) gave rise to colonies of megakaryocytes in *in vitro* cultures [8 10 11] Also the presence of red cell precursors (BFU-E) and their more mature progeny (CFU-E) was disclosed by the formation of erythrocyte colonies [5 6, 25]

T lymphocyte (CFU-TL) and B lymphocyte (CFU-BL) progenitor cells which seem to be relatively more mature cells were found to have the ability to undergo clonal proliferation in systems containing stimulatory mitogens or conditioned medium. In this system, T lymphocyte colony formation requires that the seeded cells undergo T mitogen stimulation, i.e. phytohemagglutinin (PHA) or concanavalin A [15 16, 18, 22] while B lymphocyte colony growth necessitates sensitization by one of the following: mercaptoethanol [9] a humoral thymic factor [21] or polyclonal B cell activators [17 24] Stimulating a human B-cell-enriched population with pokeweed mitogen or PHA generated the appropriate signal for triggering B lymphocyte precursors into B cell colony formation [14]

The cloning potential of T lymphocytes was significantly enhanced by supplementing the soft agar culture with lymphocyte culture fluid (Ly-CF) obtained from mitogen-treated lymphocytes [28] It was inhibited by the addition of monocyte-macrophage culture fluid (M-CF) or spleen culture fluid (SpCF [–3 38]). Differences in colony growth conditions and requirements and colony plating efficiency have been reported [3 4 15 18]

In order to establish the presence of a mechanism regulating lymphocyte colony growth, we studied the colony-forming ability of T cells as manifested by the proliferation and colony formation of PHA-stimulated lymphocytes, using a two-step method and of untreated lymphocytes, employing a one-step procedure.

Materials and Methods

Isolation of Cells

Lymphocytes, purified T cells, mononuclear cells and adherent cells were obtained from human venous blood. Blood from healthy volunteers was drawn into disposable plastic syringes containing 250–500 IU heparin (pyrogen-free, Evans). Lymphocytes were isolated from the leukocyte-rich plasma on glass columns [13]. The isolated cells comprised 85" or more lymphocytes, a few nonadherent polymorphonuclear leukocytes and 1–2% monocytes. The mononuclear cells were separated from whole blood by centrifugation through Lymphoprep (sodium metrizoate Ficoll gradient (D 1.077 [1]). The cell suspension was washed 3 times and resuspended in Eagle's medium (EM Dulbecco's modified; Gibco). The isolated mononuclear cells comprised 80–85" lymphocytes and 15–20% monocytes. T-lymphocyte-enriched population was prepared from mononuclear cells by E-rosetting these cells with sheep red blood cell (SRBC) receptors and separating them by density sedimentation through Lymphoprep. The E-rosetted cells settled in a pellet at the bottom of the tube, were removed and subjected to a 7-min treatment with NH Cl (0.8%) whereas the SRBC lysed. The remaining T cell population was washed twice with EM

Blood-adherent cells were isolated from mononuclear cell suspensions by adhering them to plastic dishes in the presence of 70% pooled human serum (HS). After h of incubation at 37 C the dishes were washed with EM to remove the nonadherent cells [28]. The adherent cells were dislodged from the plastic surface with a rubber policeman and the viable cells were scored.

Spleen Cells

Human spleen cells were obtained from patients splenectomized because of traumatic rupture of the spleen [23].

Depletion of Phagocytic Cells

Mononuclear cell suspensions were depleted of phagocytes by the carbonyl iron technique [7]. ... mononuclear cells (15 × 10⁶) were incubated 1 h at 37 C in 2 ml EM containing 10%/ HS ... 20–30 mg carbonyl iron; the suspension was ...d by shaking every 15 min. The non-iron-...ng cells were separated magnetically from ... had ingested iron, washed 3 times with ... and the viable cells counted

Preparation of Ly-CF M-CF and Sp-CF

Blood lymphocytes (1.5 × 10⁶ cells/ml) were ...tured for 3 days in the presence of PHA-M 0125 ml/ml medium) and 10%/ HS. The suspen... was centrifuged, the supernatant retained and ... at 20 °C [23 28] Blood mononuclear ... (4 × 10⁶/ml) or spleen cells (10⁷/ml) that adhered to plastic dishes were cultured in ... The cell-free medium collected after an incu...tion period of 4 days was used

Soft Agar Culture

T... culture methods were employed

T o-Step Method. The isolated cells were sen...ed ...th PHA (0 0125 ml/ml) in ... liquid cul... phase for 18 h at 37 °C in 5–7 5% CO in air subsequently seeded in ... two-layer soft-agar ...ture system containing PHA [16] Liquid cul... of phagocyte-depleted lymphocytes or from cell population reconstituted with adherent ... to concentrations of 3, 5, 10, 15 or 30% were ... When Ly-CF M-CF or Sp-CF were used in ... culture system, the EM of the lower agar layer ... replaced by the specific culture fluid The cul... dishes were incubated at 37 C in ... fully ...idified atmosphere containing 5–7 5%/ CO in ... Colonies were observed and scored under an ... l microscope after 5–6 days of culture.

One-Step Method Cells not subjected to treat... with PHA were plated directly in the agar ...ture system to ... hich Ly-CF had, or had not, ... added Colonies were examined and counted 6–7 days of cultures Colonies containing ...re than 50 cells were scored, discrete groups of ... 50 cells were tabulated as clusters.

Morphologic Studies

The large colonies growing ... ithin the upper layer were aspirated with capillary tubes, ... to slides, mixed with inactivated HS and dried. The small flat colonies that formed on the surface of the upper layer were harvested by adding 0.5 ml EM to the culture plate, removing them with ... Pasteur pipet and cytocentrifuging them onto slides. The slides were stained with May-Grünwald-Giemsa and the morphology of the cell examined.

Lymphoid C ll Surface Markers

Pooled colonies were suspended in EM the cells were tested for E-rosette-forming capacity [27] and for immunoglobulin-bearing cell content [26]

Results

Two-Step Culture Method

When human mitogen-sensitized lymphocytes were cultured in a two-layer soft agar culture system in the presence of PHA, they proliferated and developed into colonies. Two distinct types of colonies de veloped in the upper agar layer: type I large colonies of 50–500 or more cells which appeared within the upper layer 3–4 days after the cells were seeded, and type II, small flat colonies composed of 50–200 cells which formed on the surface of the upper layer after 6–7 days of culture (fig. 1 2).

When the lower agar layer was supple mented with Ly-CF the cloning potential of PHA-treated lymphocytes was enhanced by 70–100% (table I fig. 3).

If human blood mononuclear cells were seeded instead of isolated lymphocytes, the cloning efficiency improved by more than 100%. Adding Ly-CF to the soft-agar cul ture of seeded mononuclear cells augmented the number of colonies by 700% (table I). The highest cloning efficiency (1...50) was obtained when blood mononuclear cells were cultured in the presence of Ly-CF

When the agar culture system was supplemented with M-CF or Sp-CF an inhibit

colonies containing human eosinophils were observed adjacent to monocyte-macrophage colonies [19]. Progenitor cells (CFU-M) gave rise to colonies of megakaryocytes in *in vitro* cultures [8, 10, 11]. Also the presence of red cell precursors (BFU-E) and their more mature progeny (CFU-E) was disclosed by the formation of erythrocyte colonies [5, 6, 25].

T lymphocyte (CFU TL) and B lymphocyte (CFU BL) progenitor cells which seem to be relatively more mature cells were found to have the ability to undergo clonal proliferation in systems containing stimulatory mitogens or conditioned medium. In this system, T lymphocyte colony formation requires that the seeded cells undergo T mitogen stimulation, i.e. phytohemagglutinin (PHA) or concanavalin A [15, 16, 18, 22], while B lymphocyte colony growth necessitates sensitization by one of the following: mercaptoethanol [9], a humoral thymic factor [21], or polyclonal B cell activators [17, 24]. Stimulating a human B-cell-enriched population with pokeweed mitogen or PHA generated the appropriate signal for triggering B lymphocyte precursors into B cell colony formation [14].

The cloning potential of T lymphocytes was significantly enhanced by supplementing the soft agar culture with lymphocyte culture fluid (Ly-CF) obtained from mitogen-treated lymphocytes [28]. It was inhibited by the addition of monocyte macrophage culture fluid (M-CF) or spleen culture fluid (SpCF [23, 38]). Differences in colony growth conditions and requirements and colony plating efficiency have been reported [3, 4, 15, 18].

In order to establish the presence of a mechanism regulating lymphocyte colony growth, we studied the colony forming ability of T cells as manifested by the proliferation and colony formation of PHA-stimulated lymphocytes, using a two-step method and of untreated lymphocytes, employing a one-step procedure.

Materials and Methods

Isolation of Cells

Lymphocytes, purified T cells, mononuclear cells and adherent cells were obtained from human venous blood. Blood from healthy volunteers was drawn into disposable plastic syringes containing 250–500 IU heparin (pyrogen-free, Evans). Lymphocytes were isolated from the leukocyte-rich plasma on glass columns [13]. The isolated cells comprised 85% or more lymphocytes, a few nonadherent polymorphonuclear leukocytes and 3–4% monocytes. The mononuclear cells were separated from whole blood by centrifugation through Lymphoprep (sodium metrizoate/Ficoll) gradient (D 1.077) [1]. The cell suspension was washed 3 times and resuspended in Eagle's medium (EM Dulbecco's modified, Gibco). The isolated mononuclear cells comprised 80–85% lymphocytes and 15–20% monocytes. T-lymphocyte-enriched population was prepared from mononuclear cells by E-rosetting those cells with sheep red blood cell (SRBC) receptors and separating them by density sedimentation through Lymphoprep. The E-rosetted cells settled in pellet at the bottom of the tube, were removed and subjected to a 2-min treatment with NH_4Cl (0.8%) wherein the SRBC lysed. The remaining T cell population was washed twice with EM.

Blood-adherent cells were isolated from mononuclear cell suspensions by adhering them to plastic dishes in the presence of 20% pooled human serum (HS). After 2 h of incubation at 37 °C, the dishes were washed with EM to remove the nonadherent cells [28]. The adherent cells were dislodged from the plastic surface with a rubber policeman and the viable cells were scored.

Spleen Cells

Human spleen cells were obtained from patients splenectomized because of traumatic rupture of the spleen [23].

Depletion of Phagocytic Cell

Mononuclear cell suspensions were depleted of agocytes by the carbonyl iron technique [7]. mononuclear cells (15×10^6) were incubated 1 h at 37 °C in 2 ml EM containing 10% HS d 20–30 mg carbonyl iron, the suspension was by shaking every 15 min. The non-iron-aining cells were separated magnetically from that had ingested iron, washed 3 times with and the viable cells counted.

Preparation of Ly-CF, M-CF and Sp-CF

Blood lymphocytes (1.5×10^6 cells/ml) were tured for 3 days in the presence of PHA-M ml/ml medium) and 10% HS. The suspen- was centrifuged, the supernatant retained and ed at −20 °C [23, 28]. Blood mononuclear s (4×10^6/ml) or spleen cells (10^7/ml) that adhered to plastic dishes were cultured in The cell-free medium collected after an incu- ion period of 4 days was used.

oft Agar Culture

culture methods were employed.

o-Step Method. The isolated cells were sen- ith PHA (0.0125 ml/ml) in a liquid cul- phase for 18 h at 37 °C in 5–7.5% CO_2 in air subsequently seeded in two-layer soft-agar ure system containing PHA [16]. Liquid cul- of phagocyt-depleted lymphocytes or from cell population reconstituted with adherent to concentrations of 3, 5, 10, 15 or 30% were When Ly-CF, M-CF or Sp-CF were used in culture system, the EM of the lower agar layer replaced by the specific culture fluid. The cul- dishes were incubated at 37 °C in fully midified atmosphere containing 5–7.5% CO_2 in Colonies were observed and scored under an l microscope after 5–6 days of culture.

Step Method. Cells not subjected to treat with PHA were plated directly in the agar system to which Ly-CF had, or had not, added. Colonies were examined and counted 6–7 day of cultures. Colonies containing than 50 cells were scored: discrete groups of 0 cells were tabulated as clusters.

orphologic Studies

large colonies growing within the upper layer were aspirated with capillary tubes, sferred to slides, treated with inactivated HS and dried. The small flat colonies that formed on the surface of the upper layer were harvested by adding 0.5 ml EM to the culture plate, removing them with Pasteur pipet and cytocentrifuging them onto slides. The slides were stained with May-Grünwald-Giemsa and the morphology of the cells examined.

Lymphoid Cell Surface Markers

Pooled colonies were suspended in EM; the cells were tested for E-rosette-forming capacity [27] and for immunoglobulin-bearing cell content [26].

Results

Two-Step Culture Method

When human mitogen-sensitized lymphocytes were cultured in a two-layer soft agar culture system in the presence of PHA, they proliferated and developed into colonies. Two distinct types of colonies developed in the upper agar layer: type I, large colonies of 50–500 or more cells which appeared within the upper layer 3–4 days after the cells were seeded, and type II small flat colonies composed of 50–200 cells which formed on the surface of the upper layer after 6–7 days of culture (fig. 1, 2).

When the lower agar layer was supplemented with Ly-CF, the cloning potential of PHA-treated lymphocytes was enhanced by 70–100% (table I, fig. 3).

If human blood mononuclear cells were seeded instead of isolated lymphocytes, the cloning efficiency improved by more than 100%. Adding Ly-CF to the soft-agar culture of seeded mononuclear cells augmented the number of colonies by 200% (table I). The highest cloning efficiency (1:50) was obtained when blood mononuclear cells were cultured in the presence of Ly-CF.

When the agar culture system was supplemented with M-CF or Sp-CF, an inhibit



Fig. 1. Unstained, type I colonies from human peripheral blood which grew within the upper agar layer 4–5 days after seeding using the two-step PHA culture method.



Fig. 2. Unstained, small, flat type II colon which grew on the surface of the upper agar la 6–7 days after seeding, employing the one-st PHA and lymphocyte culture fluid method.

Table I. Influence of Ly-CF on T lymphocyte colony formation in the one and two-step soft-agar cultu systems

Cells seeded 5×10^4	Soft-agar culture	Number of colonies formed			
		one-step method		two-step method	
		type I	type II	type I	type II
Isolated lymphocytes	EM	0	0	100 ± 15	1,000 ± 200
	Ly-CF	60 ± 20	1 106 ± 267	180 ± 20	~2,000*
Mononuclear cells	EM	0	clusters	229 ± 21	1 100 ± 182
	Ly-CF	64 ± 18	1 100 ± 200	307 ± 52	~4,000*

The surface of the upper layer was completely covered with colonies making their exact number difficult to score.

ing effect was observed (fig. 3) The effect was proportional to the amount of culture fluid substituted for EM in the lower agar layer at 100% substitution 70% inhibition was attained (fig. 3) When E rosette-forming cells were seeded, the number of colonies formed was slightly lower than when the isolated lymphocytes were plated

Influence of Peripheral Blood Adherent Cells on T Lymphocyte Colony Formation.

Depleting mononuclear cell suspension of phagocytic cells prior to liquid culture reduced the number of colonies by 88%. We found that adding 15 % adherent cells completely restored colony formation, raising it

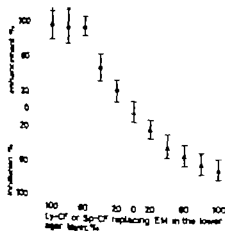


Fig. 3. Ly-CF (O) which contained LCEF and -CF (△) with its LCIF respectively stimulated and inhibited lymphocyte colony growth

that of an undepleted cell population the ion of 30% adherent cells inhibited colony formation by 45%.

One Step Culture Method

When plated directly in a two-layer soft agar culture system supplemented with PHA and Ly-CF isolated lymphocytes or mononuclear cells proliferated and developed into colonies. However in the absence of Ly-CF no colonies formed (table I). comparing the morphology of type I colonies formed in the one- and two-step methods, we noted that those from the one-step method did not have dense compact centers, but rather a diffuse structure. Type II colonies from both methods had similar morphology

Morphology and Surface Markers

The colonies consisted of large pyroninophilic blast-like cells with prominent nuclei. Mitotic forms were seen. Approximately 75% of the colony cells were E-rosette-forming: no significant immunofluorescence was detected when surface immunoglobulin receptors were sought.

Discussion

The purpose of this report is to summarize our studies on the interactions of hemopoietic cells and/or their products in a conditioned medium, and how these influence T lymphocyte growth by acting on the cell cycle of a particular population of circulating cells. Our two- and one-step cultures systems facilitated an extensive examination of factors affecting T lymphocyte colony formation and thus contributed to our understanding of this phenomenon.

In an earlier investigation, we showed that when lymphocytes sensitized with PHA in liquid phase (step 1) were plated in the presence of mitogen in a two-layer soft agar culture system (step 2), a circumscribed population of the mitogen-treated lymphocytes proliferated and developed into colonies even though no specific humoral colony stimulating factor had been supplemented to the culture [16]. When Ly-CF was added to the system, T cell colony formation was markedly enhanced and the number of type I colonies doubled (table I). These data clearly indicated that PHA-stimulated lymphocytes produced and secreted into the culture medium their own colony-stimulating factor a lymphocyte-colony-enhancing factor (LCEF), that stimulated the development and growth of T cell colonies. The presence of the mitogen in the soft agar culture is apparently necessary for maintaining the processes of lymphocyte blast transformation and secretion of the requisite quantities of LCEF. Preliminary experiments re

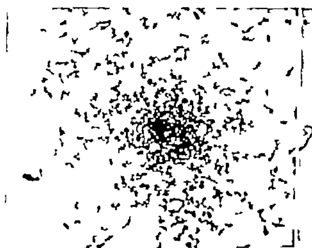


Fig. 1 Unstained, type I colonies from human peripheral blood which grew within the upper agar layer 4–5 days after seeding using the two-step PHA culture method.



Fig. 2. Unstained, small, flat type II colonies which grew on the surface of the upper agar layer 6–7 days after seeding, employing the one-step PHA and lymphocyte culture fluid method.

Table I Influence of Ly-CF on T lymphocyte colony formation in the one- and two-step soft-agar culture systems

Cells seeded 5×10^3	Soft-agar culture	Number of colonies formed			
		one-step method		two-step method	
		type I	type II	type I	type II
Isolated lymphocytes	EM	0	0	100 ± 15	1,000 ± 200
	Ly-CF	60 ± 20	1 106 ± 267	180 ± 20	~2,000*
Mononuclear cells	EM	0	clusters	229 ± 21	1 100 ± 182
	Ly-CF	64 ± 18	1 100 ± 200	307 ± 5	~2,000*

The surface of the upper layer was completely covered with colonies making their exact number difficult to score.

ing effect was observed (fig 3) The effect was proportional to the amount of culture fluid substituted for EM in the lower agar layer at 100% substitution 70% inhibition was attained (fig. 3). When E rosette-forming cells were seeded, the number of colonies formed was slightly lower than when the isolated lymphocytes were plated.

Influence of Peripheral Blood Adherent Cells on T Lymphocyte Colony Formation

Depleting mononuclear cell suspensions of phagocytic cells prior to liquid culture reduced the number of colonies by 88%. We found that adding 15 / adherent cells completely restored colony formation, raising it

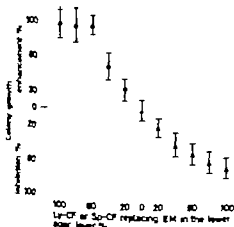


Fig. 3. Ly-CF (O) which contained LCEF and Sp-CF (△) with its LCIF respectively stimulated inhibited lymphocyte colony growth

to that of an undepleted cell population the addition of 30% adherent cells inhibited colony formation by 45 %.

One Step Culture Method

When plated directly in a two-layer soft agar culture system supplemented with PHA and Ly-CF isolated lymphocytes or mononuclear cells proliferated and developed into colonies. However in the absence of Ly-CF no colonies formed (table I). When comparing the morphology of type I colonies formed in the one- and two-step methods, we noted that those from the one-step method did not have dense compact centers, but rather a diffuse structure. The type II colonies from both methods had similar morphology

Morphology and Surface Markers

The colonies consisted of large pyroninophilic blast like cells with prominent nucleoli. Mitotic forms were seen. Approximately 75% of the colony cells were E-rosette forming: no significant immunofluorescence was detected when surface immunoglobulin receptors were sought.

Discussion

The purpose of this report is to summarize our studies on the interactions of hemopoietic cells and or their products in a conditioned medium, and how these influence T lymphocyte growth by acting on the cell cycle of a particular population of circulating cells. Our two- and one-step cultures systems facilitated an extensive examination of factors affecting T lymphocyte colony formation and thus contributed to our understanding of this phenomenon.

In an earlier investigation, we showed that when lymphocytes sensitized with PHA in liquid phase (step 1) were plated in the presence of mitogen in a two-layer soft agar culture system (step 2), a circumscribed population of the mitogen treated lymphocytes proliferated and developed into colonies even though no specific humoral colony stimulating factor had been supplemented to the culture [16] When Ly-CF was added to the system, T cell colony formation was markedly enhanced and the number of type I colonies doubled (table I). These data clearly indicated that PHA-stimulated lymphocytes produced and secreted into the culture medium their own colony-stimulating factor a lymphocyte-colony-enhancing factor (LCEF) that stimulated the development and growth of T cell colonies. The presence of the mitogen in the soft agar culture is apparently necessary for maintaining the processes of lymphocyte blast transformation and secretion of the requisite quantities of LCEF Preliminary experiments re-

vealed this factor to be of protein nature and to have a molecular weight of 90 000–110 000 daltons [29]

Based on our experiments with the two-step method, we evolved the one-step method in which the liquid-phase stage was omitted but in which the lower agar layer was supplemented with Ly-CF containing the LCEF

The growth of T cell colonies under these conditions demonstrated that T lymphocyte colony development required a humoral factor and that this factor had been secreted by a mitogen sensitized T cell population into the Ly-CF added to soft agar medium. In the two-step method the cloning efficiency of plated mononuclear cells was greater than that of isolated lymphocytes or purified T cells. This may be due either to accelerated generation of LCEF by T cells subsequent to interactions between lymphocytes and macrophage bound PHA in the liquid phase or to the production of a lymphocyte colony promoting factor by monocytes-macrophages [23]

No differences in cloning efficiency were manifested between isolated lymphocytes and mononuclear cells in the one-step method. The plating efficiency in the two-step method was greater than in the one-step method. This may possibly reflect interactions between monocytes macrophages and lymphocytes in the liquid-phase sensitization stage.

The dependence of lymphocytes on monocytes macrophages was clearly displayed in those experiments where macrophage-depleted lymphocytes were cultured and colony formation found to be sharply reduced. The ability to proliferate and develop into colonies was restored by the addition of 15% adherent cells, approximately the monocyte content of a normal blood mononuclear suspension. However if 30% adherent cells were supplemented to the inoculum, marked inhibition was observed.

Clonal proliferation was also suppressed when spleen cell culture fluid was incorporated in the agar culture system the extent of inhibition was a function of the amount of Sp-CF introduced (fig. 3). The lymphocyte-colony-inhibiting factor (LCIF) present in monocyte macrophage culture fluid had a prostaglandin E_1 E_2 nature and a molecular weight less than 1 000 daltons [70]

Our studies on the colony growth of normal human T lymphocytes indicate the presence of a system in which stimulating and inhibiting factors exert a competitive influence on T cells and thus control and regulate clonal proliferation.

References

1 Böyum, A. Separation of lymphocytes from blood and bone marrow Scand J clin. Lab. Invest. *21* suppl. 97 (1968)

2 Bradley T R., and Metcalf, D The growth of mouse bone marrow cells *in vitro* Austr J exp Biol. med. Sci. *44* 287–300 (1966).

3 Claësson, M. H Rodger M B., Johnson, G. R. Whittingham, S., and Metcalf, D Colony formation by human T lymphocytes in agar medium. Clin. exp. Immunol. *28*. 526–534 (1977)

4 Claësson, M H Whittingham, S. Rodger M. B and Burgess, A. W Colony growth of human T lymphocytes in agar effect of a soluble factor from adherent cells. Eur J Immunol *7* 608–612 (1977).

5 Heath, D S. Axelrad, A. A., McLeod, D. L., and Shreeve, M M Separation of erythropoietin responsive progenitors BFU-E in mouse bone marrow by unit gravity sedimentation. Blood *47* 777–792 (1976).

6 Iscove, N N and Sieber F.. Macroscopic erythroid colony formation in cultures of mouse bone marrow cells. Expl Hemat. *3* 32–43 (1975)

7 Lundgren, G Zukoski, C. F and Moller G Differential effects of human granulocytes and lymphocytes on human fibroblast *in vitro* Clin. exp Immunol. *3* 817–836 (1968).

8 Metcalf, D MacDonald, H R., Odartchenko, N., and Sordat, L. B. Growth of mouse megakaryocyte colonies *in vitro*. Proc. natn. Acad. Sci. USA 72. 1744–1748 (1975).

9 Metcalf, D., Warner N L., Nossal, G J V Miller J F A. P and Shortman, K. Growth of B lymphocyte colonies *in vitro* from mouse lymphoid organs. Nature, Lond. *255* 630–632 (1975).

10 Nakeff, A. and Daniels-McQueen, S. *I vitro* colony assay for new class of megakariocytes precursor colony forming unit, megakaryocyte (CFU-M). Proc. Soc. exp. biol Med. *151* 587–590 (1976).

11 Nakeff, A Dicke, K. A., and van Noord, M. J Megakariocytes in agar cultures of mouse marrow Ser haematol. *8*. 4–21 (1975).

12 Pharnik, D H. and Sachs, L. The cloning of normal 'mast' cells in tissue culture. J cell. comp. Physiol. 66 319–324 (1965).

13 Rabinowitz, W Separation of lymphocytes on glass columns, including tissue culture observations. Blood 23 811–828 (1964).

14 Radnay J Goldman, I, and Rozenszajn, L. A. Growth of human B-lymphocyte colonies *in vitro* Nature, Lond. *278* 351 353 (1979).

15 Rion, N Bouzard, G Alcalay D., Goube de LaForest, P and Tanzer J. *I vitro* growth of colonies from human peripheral blood lymphocytes stimulated by phytohemagglutinin. Annls Immunol. Inst. Pasteur Paris *127C*. 83–93 (1976)

16 Rozenszajn, L. A Shoham, D and Kalechman, Y Clonal proliferation of PHA-stimulated human lymphocytes in soft agar culture. Immunology 29 1041 1055 (1975)

17 Rozenszajn, L. A Michlin, H Kalechman, Y and Sredni, B Colony growth *in vitro* of mitogen stimulated mouse B-lymphocytes. Immunology *32* 319–327 (1977).

18 Shen, J Wilson, F Shifrine, M., and Gershwin, M E. Selected growth of human T lymphocytes in single-phase semi-solid culture. J Immun. *119* 1299–1305 (1977).

19 Shoham, D; Ben-David, E., and Rozenszajn, L. A.: Cytochemical and morphological identification of macrophages and eosinophils in tissue cultures of normal human bone marrow Blood *44* 221 233 (1974).

20 Sredni, B. A study of the growth of murine T and B lymphocyte colonies *in vitro* and mechanisms regulating clonal formation; PhD thesis, Bar-Ilan University Ramat-Gan (1979).

21 Sredni, B., Gopas, J and Rozenszajn, L. A. Induction of B lymphocyte colony growth *in vitro* by thymus-derived stimulating factor Eur J Immunol. *8* 681–685 (1978).

22 Sredni, B., Kalechman, Y Michlin, H., and Rozenszajn, L. A. Development of colonies *in vitro* of mitogen-stimulated mouse T-lymphocytes. Nature, Lond. 259 130–132 (1976).

23 Sredni, B Michlin, H; Kalechman, Y and Rozenszajn, L. A. Regulatory influences of macrophages-secreted factors on T-lymphocyte colony growth. Cell Immunol. 36. 15–27 (1978).

24 Sredni, B. and Rozenszajn, L. A.: Relation between structure of bacterial lipopolysaccharide and the stimulation of lymphocytes into colony formation. J cell. Physiol. 96. 53–62 (1978)

25 Stephenson, J R., Axelrad, A. A., McLeod, D L., and Shreeve, M. M. Induction of colonies of hemoglobin synthesizing cells by erythropoietin *in vitro* Proc. natn. Acad. Sci. USA 68. 1542 1546 (1971).

26 Wiemeyer S Bankhurst, A. D and Williams, R. C., jr. A population of human cord blood lymphocytes which generates F receptors *in vitro* Cell. Immunol. *30*: 54–65 (1977).

27 Wybran, J Chantler, S., and Fudenberg, H. H. Isolation of normal T cells in chronic lymphatic leukemia. Lancet *I*. 126–129 (1973).

28 Zeevi, A.; Goldman, I, and Rozenszajn, L. A. Stimulation and inhibition of human T-lymphocyte colony cell proliferation by hemopoietic cell factors. Cell. Immunol. 28 235–247 (1977).

29 Zeevi, A., Goldman, I, and Rozenszajn, L. A. Partial purification and characterization of the human lymphocyte colony enhancing factor (LCEF) Immunology *34* 523–531 (1978)

L. A. Rozenszajn, Department of Life Sciences, Bar-Ilan University Ramat-Gan, Israel and Department of Medical Laboratories, Meir Hospital, Kfar Saba (Israel)

vealed this factor to be of protein nature and to have a molecular weight of 90 000–110 000 daltons [29]

Based on our experiments with the two-step method we evolved the one-step method in which the liquid phase stage was omitted but in which the lower agar layer was supplemented with Ly CF containing the LCEF

The growth of T cell colonies under these conditions demonstrated that T lymphocyte colony development required a humoral factor and that this factor had been secreted by a mitogen-sensitized T cell population into the Ly-CF added to soft agar medium. In the two-step method the cloning efficiency of plated mononuclear cells was greater than that of isolated lymphocytes or purified T cells. This may be due either to accelerated generation of LCEF by T cells subsequent to interactions between lymphocytes and macrophage bound PHA in the liquid phase or to the production of a lymphocyte colony promoting factor by monocytes macrophages [23]

No differences in cloning efficiency were manifested between isolated lymphocytes and mononuclear cells in the one-step method. The plating efficiency in the two-step method was greater than in the one-step method This may possibly reflect interactions between monocytes-macrophages and lymphocytes in the liquid-phase sensitization stage.

The dependence of lymphocytes on monocytes-macrophages was clearly displayed in those experiments where macrophage-depleted lymphocytes were cultured and colony formation found to be sharply reduced. The ability to proliferate and develop into colonies was restored by the addition of 15% adherent cells, approximately the monocyte content of a normal blood mononuclear suspension. However if 30% adherent cells were supplemented to the inoculum, marked inhibition was observed.

Clonal proliferation was also suppressed when spleen cell culture fluid was incorporated in the agar culture system the extent of inhibition was a function of the amount of Sp-CF introduced (fig. 3) The lymphocyte-colony-inhibiting factor (LCIF) present in monocyte-macrophage culture fluid had a prostaglandin E_1 E_2 nature and a molecular weight less than 1 000 daltons [20]

Our studies on the colony growth of normal human T lymphocytes indicate the presence of a system in which stimulating and inhibiting factors exert a competitive influence on T cells and thus control and regulate clonal proliferation.

References

1 Bøyum, A., Separation of lymphocytes from blood and bone marrow Scand. J clin. Lab. Invest. *21* suppl 97 (1968)

2 Bradley T R. and Metcalf, D The growth of mouse bone marrow cells *in vitro* Austr J exp. Biol. med. Sci. *44* 287–300 (1966).

3 Claësson, M. H Rodger M. B. Johnson, G R. Whittingham, S., and Metcalf D Colony formation by human T lymphocytes in agar medium. Clin. exp Immunol. *28* 526–534 (1977).

4 Claesson, M. H. Whittingham, S., Rodger M. B and Burgess, A. W Colony growth of human T lymphocytes in agar effect of a soluble factor from adherent cells. Eur J Immunol *7* 608–612 (1977)

5 Heath, D S., Axelrad, A. A., McLeod, D. L., and Shreeve, M. M., Separation of erythropoietin-responsive progenitors BFU E in mouse bone marrow by unit gravity sedimentation. Blood *47* 777–792 (1976).

6 Iscove, N N and Sieber F Macroscopic erythroid colony formation in cultures of mouse bone marrow cells. Expl Hemat. *3* 32–43 (1975)

Abrams et al. for studies on granulopoiesis, mainly for an easy and rapid electronic monitoring and counting of colonies using their light scattering properties. For unknown reasons, however the method since that time was virtually abandoned in hematology We have adopted and substantially refined the method of culturing hematopoietic cells in agar capillaries. We determined the optimal conditions for growing mouse granulocytes and macrophages [*Maurer and Henry* 1976a, 1978] human peripheral T lymphocytes [*Maurer et al.*, 1977] mouse spleen B lymphocytes [*Ulmer and Maurer* 1978] as well as myelomonocytes from human bone marrow [*Maurer et al.*, 1979a]

In essence, we make use of glass capillary tubes having 1.38 mm internal diameter and 126 mm length which are filled with 75 μl of cell suspension from mouse (7.5×10^4 cells/capillary) or 50 μl human bone marrow (3.5×10^4 cells/capillary) together with an optimal type and quantity of serum and CSF The tube dimensions are such that CO_2 *and O* exposure is sufficient to guarantee homogenous growth along the gel. Methylcellulose appears to be inferior to agar, since the suspended cells settle to the tube bottom and do not form distinct colonies as seen in the agar We found that *mouse lung conditioned medium stimulates* mainly differentiation of the progenitor cells to granulocytes with low percentages of macrophages. In fact, colonies with 95% granulocytes can be obtained under appropriate conditions. On the average we count about 20 colonies (aggregates > 50 cells) from mouse bone marrow and up to 50 colonies from human bone marrow after 7 days of culture. Horse serum (30% and 20%) is essential for good growth in the mouse and human capillary assay respectively Human bone marrow stem cells are best isolated from the middle layer of a Ficoll-Isopaque gradient after low speed centrifugation.

T lymphocyte colonies are cultured from human peripheral cells isolated by centrifugation in a Ficoll-Isopaque gradient [*Boyum*, 1968] and prestimulated with PHA for 24 h prior to seeding with agar and fresh PHA into the capillaries. In 30 μl of final lymphocyte suspension with 4 400 cells per capillary we count about 60 colonies after 7 days of culture. Compact colonies of T cells develop along the tube bottom. It is important to note that colony yield depends mainly on the seeded cell density with a sharp optimum at 2×10^4 cells/ml gel irrespective of its length. Plating efficiencies are 10- to 50-fold higher in the capillaries than in Petri dishes.

The tube-to-tube coefficient of variation does not exceed 10% for the granulocyte assay or 5% for the T lymphocyte assay In general, a final agar concentration of only 0.18% was found to be necessary for optimal growth in these assays.

Optimal conditions were also established for the production of B lymphocyte colonies from mouse spleen cells in the capillaries using a bacterial lipopolysaccharide as mitogen [*Ulmer and Maurer* 1978]. In addition, 5×10^{-4} *M mercaptoethanol and* 20% horse serum are needed and an agar concentration of 0.3% is best for colony counting. A sigmoid curve relating the number of colonies with the seeded cell density suggests that cell-to-cell cooperation is essential for colony formation.

The linear alignment of the colonies along the gel greatly facilitates colony counting. Moreover we developed an optical, semi-automatic scanning system, on the

Acta haemat. 62 322–325 (1979)

In vitro Colony Growth of Granulocytes, Macrophages, T and B Lymphocytes in Agar Capillaries

H. Rainer Maurer

Pharmazeutisches Institut, Freie Universität Berlin, Berlin

Key Words. Agar capillaries · B lymphocytes · Colonies · Granulocytes · Macrophages · T lymphocytes

Abstract. Compared with the common agar culture of hematopoietic colonies in Petri dishes, the use of glass capillary tubes offers a couple of advantages which have been exploited. Among these advantages are less materials, less cells to be seeded, lower costs, easier and quicker colony counting by visual and semi-automated optical scanning, and lower risk of bacterial contamination. In tubes several parameters affecting the colony formation of mouse and human granulocytes and macrophages as well as of human peripheral T and B lymphocyte from mouse spleen were optimized. The applicability of the cultures to assay for inhibiting and stimulating humoral factors and cytostatics was demonstrated.

Since 1965 techniques have been developed to culture all major subclasses of hematopoietic cells *in vitro* using semi-solid media [see *Metcalf* 1977 for a general review). These techniques have had a large impact on the present concepts of the proliferation and differentiation of hematopoietic progenitor cells. The possibility to induce and manipulate colony formation of these cells *in vitro* has opened many avenues for studying the control mechanisms exerted by natural humoral factors as well as the effects of drugs. Generally the methods utilize Petri dishes into which the cells mixed with liquified agar or methylcellulose, nutrient medium and some source of colony stimulating factor (CSF) are seeded. The visual evaluation of colonies in Petri dishes becomes laborious, time-consuming and costly when a great number of plates need to be analyzed. We were faced with this problem when using the agar colony technique to detect specific inhibitors including chalones [*Maschler and Maurer* 1978, *Kastner and Maurer* in preparation] and stimulators (CSF) of granulopoiesis [*Neumeier and Maurer* in press] isolated by biochemical separation methods.

We therefore explored the potentials of a method whereby bone marrow cells are cultured in agar kept in glass capillaries. This technique was introduced in 1973 by

Maurer, H. R. and Henry R.: Growth kinetics by *scanning of granulocytic cell colonies in glass* capillaries. Blut *34* 89–98 (1977).

Maurer H. R. and Henry R.: Drug evaluation of haemopoietic cells *in vitro*. I. A micro agar colony assay of haemopoietic cells with semi-automatic optical monitoring. Arzneimittel Forsch. *28*. 601–605 (1978).

Maurer, H. R., Henry R., and Maschler R.: Chalone inhibition of granulocyte colony growth in agar: kinetic quantitation by capillary tube scanning. Cell Tiss. Kinet. *11* 129–138 (1978).

Maurer H. R. and Laerum, O. D. Granulocyte chalone testing: a critical review. in Houck, Chalones, pp. 331 354 (North-Holland, Amsterdam 1976).

Maurer H. R., Neumeier R., Maschler, R., Bombik, B. M., and Gerhartz, H. A micromethod to culture granulocyte colonies from human bone marrow cells in agar-containing glass capillaries. (In preparation, 1979a).

Maurer H. R.; Maschler R.; Braun, R. and Dittmar W. Einfluss von zytostatischen und immunosuppressiven Methylhydrazonen auf die Myelo- und Lymphopoese *in vitro*. Z. Krebsforsch. klin. Onkol. (in press, 1979b).

Maurer H. R., Maschler R., Dietrich, R., and Goebel, B.: *In vitro* culture of lymphocyte colonies in agar capillary tubes after PHA-stimulation. J. Immunol. Methods *18*. 353–364 (1977).

Metcalf, D. Hemopoietic cells (Springer Berlin 1977).

Neumeier R., and Maurer H. R.: Bovine lung conditioned medium as source of both human and murine colony-stimulating activity. Exp Hematol., Copenh., in press (1979).

Ulmer A. and Maurer H. R. The formation of B-lymphocyte colonies in agar contained in glass capillaries. Immunology *34* 919–925 (1978).

H. Rainer Maurer, Pharmazeutisches Institut,
Freie Universität Berlin, D–1000 Berlin 33

basis of light scattering measurement, which is mainly composed of a two-mirror system, a photodensitometer and an automatic multiple sample changer loaded with a multiple tube holder [*Maurer and Henry* 1976b] The scanning system provides an easy accurate and sensitive method to follow the colony growth kinetics day by day [*Maurer and Henry* 1977 *Maurer et al.* 1978]

In addition to these advantageous features of the agar capillary method, some others have emerged. These can be summarized and listed as follows:

Less materials (cells, medium, test samples), i.e. lower costs
Re-use of sterilized capillaries
Higher plating efficiencies (for lymphocytes)
Lower agar concentration due to capillary forces, i.e. easier staining of colony cells
Linear alignment of colonies, i.e. easier and quicker colony counting, optical evaluation by light scattering densitometry
Reduced risk of contamination by bacteria and fungi.

We have used the mouse and human granulocyte/macrophage and the T lymphocyte colony method in agar capillaries as assays for the determination of inhibiting and stimulating humoral factors from various *hematopoietic* cells. The granulocyte assay in combination with the T lymphocyte assay has proved to be indispensable for screening granulocyte-specific inhibitors (chalones) isolated from rat ascites and bone marrow cells, since the widely used ^{3}H thymidine (^{3}H Tdr) uptake measurements have failed to reveal specific inhibitors [*Maschler and Maurer* 1978] Indeed, at least 14 potential artifacts of the ^{3}H Tdr method for measuring cell proliferation can be listed [*Maurer and Laerum* 1976 *Maurer* 1979] which cast serious doubts as to the biological validity of this method. Similarly Sephadex chromatography of a calf spleen extract produced *fractions* which inhibited lymphocyte colony growth but barely inhibited ^{3}H Tdr uptake by concanavalin A stimulated mouse spleen cells [*Maschler and Maurer* 1977] Moreover when cytostatic and immunosuppressive methylhydrazones are tested and compared in the different colony and ^{3}H Tdr assays, the latter appeared to be much less sensitive [*Maurer et al.*, in press, 1979b]

To date, capillary assays are somewhat limited in that no samples can be added after cell seeding into the tubes. Apart from this minor disadvantage it appears that the features listed above should indeed favor the use of the capillaries, particularly for large-scale testing and screening purposes.

References

Abrams, L., Carmeci, P. Bull, I. M., and Carbone, P. Capillary tube scanning applied to *in vitro* mouse marrow granulocyte growth. J natn. Cancer Inst. *50* 267 (1973).
Boyum, A., Separation of leukocytes from blood and bone marrow Scand. J clin. Lab Invest. *21* suppl. 97 (1968)
Maschler R. and Maurer H. R., A lymphocyte chalone assay using lymphocyte colony growth in agar capillaries. Virchows Arch. Abt. B Zellpath. *25* 345–353 (1977).
Maschler R. and Maurer H. R., Screening for granulopoiesis inhibitors (chalones) by different assays Hoppe-Seyler's Z. physiol Chem. *359* 825–834 (1978).
Maurer H. R., Potential pitfalls of ^{3}H-thymidine methods to measure cell proliferation. Cell Tiss. Kinet. (in press, 1979).
Maurer H. R. and Henry R., Colony growth and mouse bone marrow cells in agar contained in glass capillaries. Blut *33* 11–22 (1976a)
Maurer H. R. and Henry R., Automated scanning of bone marrow cell colonies growing in agar containing glass capillaries. Expl Cell Res. *103* 271–277 (1976b)

Maurer, H. R. and Henry R.: Growth kinetics by scanning of granulocytic cell colonies in glass capillaries. Blut *34* 89–98 (1977).

Maurer H. R. and Henry R.: Drug evaluation of haemopoietic cells *in vitro*. I. A micro agar colony assay of haemopoietic cells with semi-automatic optical monitoring. Arzneimittel-Forsch. *28*, 601–605 (1978).

Maurer H. R. Henry R., and Maschler R.: Chalone inhibition of granulocyte colony growth in agar: kinetic quantitation by capillary tube scanning. Cell Tiss. Kinet. *11* 129–138 (1978).

Maurer H. R. and Laerum, O. D.: Granulocyte chalone testing: a critical review. In Houck, Chalones, pp. 331 354 (North-Holland, Amsterdam 1976).

Maurer H. R. Neumeier R., Maschler R.; Bombik, B. M., and Gerhartz, H.: A micromethod to culture granulocyte colonies from human bone marrow cells in agar-containing glass capillaries. (In preparation, 1979a).

Maurer H. R., Maschler R., Braun, R. and Dittmar W.: Einfluss von zytostatischen und immunsuppressiven Methylhydrazonen auf die Myelo- und Lymphopoese *in vitro*. Z. Krebsforsch. klin. Onkol. (in press, 1979b).

Maurer H. R.; Maschler R., Dietrich, R., and Goebel, B.: *In vitro* culture of lymphocyte colonies in agar capillary tubes after PHA-stimulation. J. Immunol. Methods *18*, 353–364 (1977).

Metcalf, D.: Hemopoietic cells (Springer Berlin 1977).

Neumeier R., and Maurer H. R.: Bovine lung conditioned medium as a source of both human and murine colony-stimulating activity. Exp. Hematol., Copenh., in press (1979).

Ulmer A. and Maurer H. R.: The formation of B-lymphocyte colonies in agar contained in glass capillaries. Immunology *34* 919–925 (1978).

H. Rainer Maurer Pharmazeutisches Institut, Freie Universität Berlin, D–1000 Berlin 33

basis of light scattering measurement, which is mainly composed of a two-mirror system, a photodensitometer and an automatic multiple sample changer loaded with a multiple tube holder [*Maurer and Henry* 1976b]. The scanning system provides an easy, accurate and sensitive method to follow the colony growth kinetics day by day [*Maurer and Henry* 1977; *Maurer et al.* 1978].

In addition to these advantageous features of the agar capillary method, some others have emerged. These can be summarized and listed as follows.

Less materials (cells, medium, test samples), i.e. lower costs
Re-use of sterilized capillaries
Higher plating efficiencies (for lymphocytes)
Lower agar concentration due to capillary forces, i.e. easier staining of colony cells
Linear alignment of colonies, i.e. easier and quicker colony counting, optical evaluation by light scattering densitometry
Reduced risk of contamination by bacteria and fungi.

We have used the mouse and human granulocyte/macrophage and the T lymphocyte colony method in agar capillaries as assays for the determination of inhibiting and stimulating humoral factors from various hematopoietic cells. The granulocyte assay in combination with the T lymphocyte assay has proved to be indispensable for screening granulocyte-specific inhibitors (chalones) isolated from rat ascites and bone marrow cells, since the widely used ^{3}H-thymidine (^{3}H Tdr) uptake measurements have failed to reveal specific inhibitors [*Maschler and Maurer* 1978]. Indeed, at least 14 potential artifacts of the ^{3}H Tdr method for measuring cell proliferation can be listed [*Maurer and Laerum*, 1976; *Maurer* 1979] which cast serious doubts as to the biological validity of this method. Similarly, Sephadex chromatography of a calf spleen extract produced fractions which inhibited lymphocyte colony growth but barely inhibited ^{3}H Tdr uptake by concanavalin A stimulated mouse spleen cells [*Maschler and Maurer* 1977]. Moreover, when cytostatic and immunosuppressive methylhydrazones are tested and compared in the different colony and ^{3}H Tdr assays, the latter appeared to be much less sensitive [*Maurer et al.*, in press, 1979b].

To date, capillary assays are somewhat limited in that no samples can be added after cell seeding into the tubes. Apart from this minor disadvantage it appears that the features listed above should indeed favor the use of the capillaries, particularly for large-scale testing and screening purposes.

References

Abrams, L.; Carmeci, P.; Bull, I. M., and Carbone, P.: Capillary tube scanning applied to *in vitro* mouse marrow granulocyte growth. J. natn. Cancer Inst. *50:* 267 (1973).
Boyum, A.: Separation of leukocytes from blood and bone marrow. Scand. J. clin. Lab. Invest. *21:* suppl. 97 (1968).
Maschler, R. and Maurer, H. R.: A lymphocyte chalone assay using lymphocyte colony growth in agar capillaries. Virchows Arch. Abt. B Zellpath. *25:* 345–353 (1977).
Maschler, R. and Maurer, H. R.: Screening for granulopoiesis inhibitors (chalones) by different assays. Hoppe-Seyler's Z. physiol. Chem. *359:* 825–834 (1978).
Maurer, H. R.: Potential pitfalls of ^{3}H-thymidine methods to measure cell proliferation. Cell Tiss. Kinet. (in press, 1979).
Maurer, H. R. and Henry, R.: Colony growth and mouse bone marrow cells in agar contained in glass capillaries. Blut *33:* 11–22 (1976a).
Maurer, H. R. and Henry, R.: Automated scanning of bone marrow cell colonies growing in agar containing glass capillaries. Expl Cell Res. *103:* 271–277 (1976b).

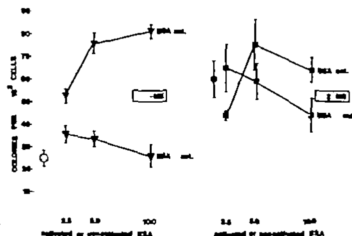


Fig. 1. The effect of addition of 2-ME, 2-ME-activated and nonactivated BSA to agar on colony growth of human T lymphocytes (2-step method). Cultures contain 5% FCS and 0.6% human AB serum. Left panel. ○ = Control cultures without BSA and without 2-ME. All cultures without 2-ME. Right panel. ● = Control cultures without BSA and with 2-ME (5×10^{-5} M). All cultures contain 2-ME (5×10^{-5} M). Data are means of quadruplicates ± SD.

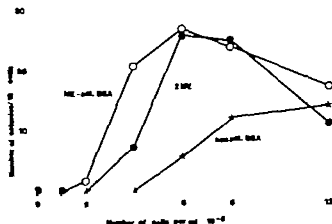


Fig. 2. The effect of addition of 2-ME, 2-ME-activated and nonactivated BSA to agar on growth of human T lymphocytes (one-step micromethod). All cultures contain 10% FCS.

or autologous serum for colony growth obtained by this method.

Preparation of Serum Factors. 1 g of bovine serum albumin (Behringwerke, Marburg, FRG) was dissolved in 10 ml of RPMI 1640 medium and 2-ME was added at a final concentration of 10^{-3} M. The preparation was incubated and dialyzed as described [4, 8]. A control preparation, not incubated with 2-ME was treated in the same way.

Adherent Cell Conditioned Medium (AC-CM). A standard batch of AC-CM was prepared from adherent blood mononuclear cells as described elsewhere [3].

Acta haemat. 62 326–330 (1979)

Role of Serum Factors and Adherent Cells in Cloning of Human T Lymphocytes in Agar Culture[1]

H D Flad A J Ulmer M H Claesson and H G Opitz

Laboratory of Immunology Department of Microbiology University of Ulm, Ulm, Anatomy Department A, University of Copenhagen, Copenhagen, and Institute for Immunology and Oncology Bayer AG, Wuppertal

Key Words. Colony formation Human T lymphocytes 2-Mercaptoethanol Serum factors

Abstract. In the presence of serum, 2 mercaptoethanol-treated bovine serum albumin enhances T cell colony formation as does 2 mercaptoethanol. The factor only partially substitutes for FCS, but neither for the mitogen phytohemagglutinin nor for conditioned medium derived from cultures of adherent cells.

Introduction

The low molecular weight thiol 2 mercaptoethanol (2 ME) has been shown to enhance antibody and mitogen induced responses of murine spleen cells *in vitro* [1 5] In systems measuring clonal proliferation of cells 2 ME enhances T lymphocyte colony formation [10] and is absolutely necessary for murine B lymphocyte colony formation [7]

Recently *Opitz et al.* [8 9] showed that a 2 ME activated serum factor (MaSF) generated from fetal calf serum or mouse serum was capable of substituting for 2 ME or macrophages and for serum in the primary antibody response of mouse spleen cells *in vitro* This factor had a molecular weight which was similar to that of albumin.

The purpose of this study was to investigate the effects of this factor on the growth of human T lymphocytes in three different systems.

Material and Methods

Cloning of Human T Lymphocytes. For growth of human T lymphocytes in agar three different methods were employed. (1) A two-step method according to *Claesson et al.* [2], in which mononuclear cells were preincubated for 18 h with PHA followed by seeding of the cells in agar (2) A one-step method, in which mononuclear cells or nonadherent cells were incorporated directly into the agar together with the mitogen PHA [3]. (3) A one-step micromethod recently developed by *Ulmer and Flad* [13] in which blood mononuclear cells are grown in capillary tubes. There is no requirement for factors, erythrocytes

[1] Supported by Deutsche Forschungsgemeinschaft, Sonderforschungsbereich 112, Project C4

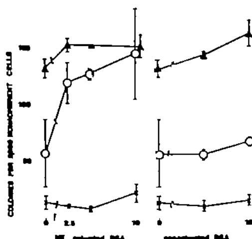


Fig. 3. The effect of activated and nonactivated BSA on colony growth of human T lymphocytes (one-step method). Nonadherent mononuclear cells are used. All cultures contain 5% FCS and 0.6% AB-serum. ▲ = Plus 2-ME plus adherent cell conditioned medium (AC-CM). O = no 2-ME plus AC-CM, X = plus 2-ME without AC-CM. Data are means of quadruplicates ± SD

stitute for the presence of AC-CM in the culture medium. Finally activated BSA could not substitute for serum in the culture medium (data not shown in figure 3).

Discussion

The results show that 2-ME-treated BSA enhances T cell colony formation similar to 2-ME. The factor was, however unable to substitute for AC-CM in the one-step method. This is in contrast to our findings in the primary antibody response *in vitro* in which 2-ME-treated fetal calf serum was capable of substituting for macrophages as well as for 2-ME [8].

Albumin and transferrin are known to be essential promoters of the growth of human lymphocytes [11, 12]. In the growth of macrophage and granulocyte colonies, these substances partially substitute for serum [6]. When adding BSA or $FeCl_3$ to FCS, *Sachs* [10] obtained similar numbers of colonies as by the addition of autologous plasma, but the results varied markedly from lectin to lectin and donor to donor [10]. Our results indicate that 2-ME-treated BSA substitutes better for serum than nontreated BSA.

References

1 Broome, J. D. and Jeng, M. W.: Promotion of replication in lymphoid cells by specific thiols and disulfides *in vitro*. J. exp. Med. *138*: 574–592 (1973).

2 Claesson, M. H.; Rodger, M. B.; Johnson, G. R.; Whittingham, S., and Metcalf, D.: Colony formation by human T-lymphocytes in agar medium. Clin. exp. Immunol. *28*: 526–534 (1977).

3 Claesson, M. H.; Whittingham, S.; Rodger, M. B., and Burgess, A. W.: Colony growth of human T-lymphocytes in agar. Effect of soluble factor from adherent cells. Eur. J. Immunol. 7: 608–612 (1977).

4 Claesson, M. H.; Fiad, H.-D., and Opitz, H. G.: B- and T-lymphocyte colony formation in agar. 2-Mercaptoethanol (2-ME) activated albumin can substitute for 2-ME. Cell Immunol. *46*: 398–404 (1979).

5 Click, R. E.; Beck, L., and Alter, B. J.: Enhancement of antibody synthesis *in vitro* by mercaptoethanol. Cell. Immunol. *3*: 156–160 (1972).

6 Guilbert, L. J. and Iscove, N. N.: Partial replacement of serum by selenite, transferrin, albumin and lecithin in haemopoietic cell cultures. Nature, Lond. *263*: 594–595 (1976).

7 Metcalf, D.: Role of mercaptoethanol and endotoxin in stimulating B-lymphocyte colony formation *in vitro*. J. Immun. *116*: 635–638 (1976).

Results

As shown in figure 1 activated BSA – which is BSA treated with 2-ME – added at the second step of the culture system, enhances the number of colonies. The enhancement is similar to that obtained by the addition of 2 ME. The control preparation – nontreated BSA – has no enhancing effect on colony growth but is also enhancing in the presence of 2 ME.

We asked the question whether or not the BSA preparations could be a substitute for serum. In table I two experiments are given. Both 2 ME-activated BSA and nonactivated BSA partially substitute for FCS in the preincubation step (table I) They do not substitute for the mitogen PHA (data not shown) On the other hand, 2 ME-activated BSA is able to substitute for FCS in the second step (= agar step) while nonactivated BSA is not. It should be noted, however that no colony growth was obtained in the complete absence of serum, i.e., in the absence of 0 6% human AB serum.

The influence of activated and nonactivated BSA was tested in a one-step micromethod which was recently developed in our laboratory [13] Activated BSA had again a strong enhancing effect on T cell colony growth which was even more pronounced than the enhancing effect of 2 ME (fig. 2)

Finally the question was asked whether activated or nonactivated BSA were able to substitute for adherent cells. This was tested in a one-step system which had been shown to depend on the presence of adherent cells or conditioned medium derived from cultures of adherent cells (AC-CM) [3]

Table I. Two-step method of human T cell colony formation

a Replacement of FCS by 2 ME activated and nonactivated BSA during preincubation with PHA[1]

Addition of	Colonies per 5 000 MNC ± SEM	
	ø 2 ME	+ 2-ME
RPMI 1640	4 ± 1	3 ± 1
BSA activated (10%)	28 ± 3	
BSA nonactivated (10%)	21 ± 3	
FCS (10%)	52 ± 3	83 ± 3

1 10^6 mononuclear cells in 1 ml were preincubated under various conditions with PHA for 18 h at 37°C and 5,000 cells were plated per Petri dish.

b Replacement of FCS by 2 ME activated and nonactivated BSA in the second step[1]

Addition of	Colonies per 5,000 MNC ± SEM	
	ø 2-ME	+ 2-ME
RPMI 1640	8 ± 1	51 ± 4
BSA activated (10%)	35 ± 3	22 ± 3
BSA nonactivated (10%)	11 ± 1	21 ± 4
FCS (10%)	26 ± 2	56 ± 4

1 10^6 mononuclear cells in 1 ml were preincubated in 10% FCS with PHA for 18 h at 37°C and 5 000 cells were plated per Petri dish under various conditions All cultures contain 0.6% human AB serum.

Mononuclear cells were depleted of adherent cells by incubation in plastic Petri dishes and the nonadherent cells were assayed for colony formation. The results in figure 3 show that 2 ME-activated BSA can substitute for 2 ME when used in a final concentration of 10%. Nonactivated BSA did not have any effect on colony formation. When AC-CM was *not* present in the culture medium colony formation was reduced by at least 90%. Neither 2 ME-activated BSA, nor nonactivated BSA nor 2 ME could sub-

Acta haemat. 62: 331-337 (1979)

Current Concepts of Abnormal Stem Cell Proliferation in Human Disease[1]

William A. Robinson

Division of Medical Oncology, Department of Medicine, University of Colorado Medical Center, Denver, Colo.

Key Words. Cell proliferation · Stem cell

Abstract. Using acute myeloid leukemia as an example, the concept of abnormal hematopoietic stem cell production has been examined. It is concluded that the major problem does not lie in humoral regulators, either stimulators or inhibitors, of stem cell production but in intrinsic cellular defects which do not allow cells to respond to these materials. The nature of the stem cell defects has not been defined. Similar mechanisms appear to exist in other human hematopoietic disorders in which abnormal stem cell proliferation is a major feature.

The human hematopoietic disorders in which abnormal stem cell proliferation is a major feature encompass a wide variety of diseases, both neoplastic and nonneoplastic. These include the leukemias, polycythemia rubra vera, agnogenic myeloid metaplasia, the lymphomas, and paroxysmal nocturnal hemoglobinuria among others. The mechanisms involved in abnormal stem cell proliferation in these disorders are probably as diverse as the diseases themselves. It is difficult to encompass them all in a single unifying concept and I will thus try to use the example of acute myeloid leukemia (AML) to cover the broad principles of probable mechanisms. The leukemias have come into particular focus over the past few years due largely to the development of *in vitro* techniques for growing and cloning human granulocyte and monocyte precursors in semi-solid tissue culture systems [2, 23-28, 30]. More recently similar systems have been developed for the clonal growth of erythroid and lymphoid cells, but these techniques have not been as widely adapted to the human hematopoietic disorders which involve these cell lines [32, 33-37].

In order to facilitate understanding of possible mechanisms of abnormal stem cell

[1] Supported by grants from the National Institutes of Health, National Cancer Institute (1R01CA23552-01), American Cancer Society CH-81, and by grant (RR 51) from the General Clinical Research Centers Program of the Division of Research Resources, National Institutes of Health.

8 Opitz, H. G., Opitz, U., Lemke, H., Hewlett, G Schreml, W., and Flad, H. D The role of fetal calf serum in the primary immune response *in vitro* J exp Med. *145* 1029–1038 (1977).
9 Opitz, H. G Opitz, U Lemke, H. Flad, H. D Hewlett, G and Schlumberger H. D Humoral primary immune response *in vitro* in a homologous mouse system. Replacement of fetal calf serum by a 2 mercaptoethanol or macrophage activated fraction of mouse serum. J Immun. *119* 2089–2094 (1977).
10 Sachs, L. Control of cloning of normal human T-lymphocytes by transferrin, albumin and different lectins. Clin. exp Immunol. *33* 495–498 (1978)
11 Spicker Polet, H. and Polet, H. Identification of albumin as the serum factor essential for the growth of activated human lymphocytes. J. biol. Chem. *251* 987–992 (1976).
12 Tormey D C., Imrie, R. C., and Mueller, G. C.. Identification of transferrin as a lymphocyte growth promoter in human serum. Expl Cell Res. *74* 163–169 (1972).
13 Ulmer A. J and Flad, H. D One-stage stimulation of human T lymphocyte colony forming units (TL-CFU) in a micro agar culture in glass capillaries. Immunology *38*. 393–399 (1979).

H. D Flad, Laboratory of Immunology
Department of Microbiology
University of Ulm, Ulm (FRG)

This would seem to be the most hopeful possibility for the treatment and correction of these diseases. Conceivably if such defects could be demonstrated, one could simply supply the missing or abnormal regulatory factors, analogous to the treatment of diabetes mellitus. Unfortunately no current evidence exists for such mechanisms in human AML. *Metcalf* [23] has recently reviewed the subject in great detail. His studies, and others, have shown that serum and urine from patients with AML have slightly higher levels of CSF than found in similar samples from normal humans, when tested on mouse bone marrow cells [24]. The colonies stimulated are, however, morphologically similar in all respects to those found using normal human material as the stimulatory source. Likewise, feeder layers derived from the peripheral blood or bone marrow of patients with most forms of AML appear to stimulate normal colony formation by normal human bone marrow cells *in vitro* [16, 30]. As with serum, the colonies formed are morphologically similar to those stimulated using normal human white blood cell feeder layers. Thus, there is no clear-cut evidence for abnormal CSF production or activity in AML.

The data concerning inhibitors is much less clear, due in a large part to the uncertain nature of the majority of these materials. *Metcalf* [23] and *Metcalf et al.* [24] have noted that about half the serum from patients with AML have subnormal or undetectable inhibitor levels when assayed on mouse bone marrow cells compared to serum from normal humans. When these sera were tested on human bone marrow cultures, however, some examples of AML serum with strong inhibitory activity were demonstrated. These inhibitory effects were presumably due to the high density lipoproteins present in human serum. Other inhibitors have been derived from mature granulocytes, which at high concentrations inhibit normal granulocyte colony growth by inhibiting the production of CSF [4, 22, 35]. No definitive studies on the levels of these materials in AML, however, have been recorded. Thus, as with CSF, there is little or no evidence at the present time that abnormalities in inhibitory factors play a major or pathophysiologic role in abnormal stem cell proliferation in AML.

It has been clearly demonstrated by a number of authors [7, 23] that peripheral blood and bone marrow blast cells from patients with AML can inhibit normal granulocyte colony formation *in vitro*. The nature of this inhibitory effect has not been determined. While these findings may explain the absence of normal hematopoiesis in such patients, they do not, at the present time, give clues as to the nature of the stem cell defect in the leukemic cells themselves. A search for abnormal inhibitory factors in the serum of patients with AML has been carried out by *Mangalik and Robinson* [21]. In this study, serum from patients with AML was tested on normal human bone marrow and compared with normal serum. No evidence of abnormal inhibition of normal granulocyte colony formation when stimulated by normal feeder layers was found.

The conclusion from all of the above findings at present is that no demonstrable pathophysiologic defects exist in the currently recognized granulocyte regulatory factors in AML which can explain abnormal stem cell proliferation in this disorder. We must then focus on the granulocyte stem cell itself. If the regulatory factors themselves are not abnormal, the stem cell itself

proliferation in human acute myeloid leukemia, some discussion of our current concepts of the regulation of granulocyte production is in order This will serve as the framework for the discussion to follow Full understanding of the regulation of granulopoiesis is not, as yet, complete but a tentative scheme, based largely on *in vitro* data, has been developed. The major positive stimulus for granulocyte production in humans appears to be the so-called colony stimulating factor (CSF) a glycoprotein with a molecular weight of approximately 45 000 daltons [23] This material stimulates committed granulocyte stem cells to divide and mature *in vitro* leading to colony formation in semi-solid tissue culture systems. There is circumstantial evidence that CSF serves a similar positive stimulatory role *in vivo* [23 26] The major source of this material in humans appears to be the monocyte-macrophage system, although lymphocytes have also been implicated in its production [6 8 15 20 34] The factors regulating CSF production by these cell lines have not been totally worked out, but it has been shown that microorganisms and/or their products enhance CSF production both *in vivo* and *in vitro* [14 20 31] Thus, the positive or stimulatory arm in granulocyte production appears to be mediated through CSF with the latter modulated, at least in part, by levels of microorganisms. The negative feedback arm in the regulation of granulopoiesis is much less clear Work from our own laboratory has suggested that this is accomplished through inactivation of the microorganism stimulus for CSF production [20 31] Other authors have, however presented considerable evidence for the presence of inhibitory factors in serum, or made by granulocytes themselves [4 17 22, 35] The mechanism of action of these inhibitors is variable with some acting directly on proliferating cells while others appear to interfere with CSF production. The final component in the granulocyte regulatory scheme is the stem cell itself. In the *in vitro* systems in which granulocyte production has been studied, the colony forming cell appears to be a committed stem cell which is able to differentiate into both the granulocyte and monocyte-macrophage lines [23] The mechanism of action of CSF and the above noted inhibitory factors on this committed granulocyte precursor cell *in vitro* and *in vivo* have not been determined.

Thus the overall system in the regulation of granulocyte production has three reasonably defined component parts – the stimulatory arm mediated by CSF the responsive granulocyte stem cell and a poorly defined inhibitory arm. It is likely that similar mechanisms, already partially defined, exist for other hematopoietic stem cell lines.

Using this schematic outline, the following possibilities are evident for abnormal stem cell proliferation in acute myeloid leukemia, the model to be discussed here. (1) Quantitative or qualitative abnormalities in the humoral regulatory factors (stimulators and inhibitors) (2) Intrinsic granulocyte stem cell defects in which these cells are no longer able to respond to these regulatory factors (3) Also to be considered is the possibility that extrinsic, or new stimulators or inhibitors exist in this disorder which interfere with the action of the normal humoral regulators. Let us consider these possibilities one by one.

It has long been hoped that the human leukemias might represent simple qualitative or quantitative defects in the humoral factors regulating granulocyte production.

This would seem to be the most hopeful possibility for the treatment and correction of these diseases. Conceivably if such defects could be demonstrated, one could simply supply the missing or abnormal regulatory factors, analogous to the treatment of diabetes mellitus. Unfortunately no current evidence exists for such mechanisms in human AML. *Metcalf* [23] has recently reviewed the subject in great detail. His studies, and others, have shown that serum and urine from patients with AML have slightly higher levels of CSF than found in similar samples from normal humans, when tested on mouse bone marrow cells [24] The colonies stimulated are, however morphologically similar in all respects to those found using normal human material as the stimulatory source. Likewise, feeder layers derived from the peripheral blood or bone marrow of patients with most forms of AML appear to stimulate normal colony formation by normal human bone marrow cells *in vitro* [16, 30] As with serum, the colonies formed are morphologically similar to those stimulated using normal human white blood cell feeder layers. Thus, there is no clear-cut evidence for abnormal CSF production or activity in AML.

The data concerning inhibitors is much less clear due in a large part to the uncertain nature of the majority of these materials. *Metcalf* [23] and *Metcalf et al.* [24] have noted that about half the serum from patients with AML have subnormal or undetectable inhibitor levels when assayed on mouse bone marrow cells compared to serum from normal humans. When these sera were tested on human bone marrow cultures, however, some examples of AML serum with strong inhibitory activity were demonstrated. These inhibitory effects were presumably due to the high density lipoproteins present in human serum. Other inhibitors have been derived from mature granulocytes, which at high concentrations inhibit normal granulocyte colony growth by inhibiting the production of CSF [4 22, 35] No definitive studies on the levels of these materials in AML, however have been recorded. Thus, as with CSF there is little or no evidence at the present time that abnormalities in inhibitory factors play a major or pathophysiologic role in abnormal stem cell proliferation in AML.

It has been clearly demonstrated by a number of authors [7 23] that peripheral blood and bone marrow blast cells from patients with AML can inhibit normal granulocyte colony formation *in vitro* The nature of this inhibitory effect has not been determined. While these findings may explain the absence of normal hematopoiesis in such patients, they do not, at the present time, give clues as to the nature of the stem cell defect in the leukemic cells themselves. A search for abnormal inhibitory factors in the serum of patients with AML has been carried out by *Mangalik and Robinson* [21]. In this study serum from patients with AML was tested on normal human bone marrow and compared with normal serum. No evidence of abnormal inhibition of normal granulocyte colony formation when stimulated by normal feeder layers was found.

The conclusion from all of the above findings at present is that no demonstrable pathophysiologic defects exist in the currently recognized granulocyte regulatory factors in AML which can explain abnormal stem cell proliferation in this disorder. We must then focus on the granulocyte stem cell itself. If the regulatory factors themselves are not abnormal, the stem cell itself

must lack the ability to respond in a normal fashion All of the available human data suggest that this is indeed the case In animal systems, there is considerable evidence that various leukemia cell lines can be induced to proliferate and differentiate *in vitro* in the presence of appropriate stimuli. *Ichikawa* [19] demonstrated that cells from a transplantable mouse myeloid leukemia which did not differentiate in liquid suspension cultures, could be induced to form colonies with a high proportion of mature granulocytes and monocytes in the presence of mouse embryo conditioned medium. *Fibach et al.* [10] and *Fibach and Sachs* [11 12] confirmed and extended these findings in the same cell line, and demonstrated that these cells lost their leukemia-inducing capacity *in vivo* if preincubated with mouse lung conditioned medium. *Metcalf* [23] and *Metcalf and Moore* [25] demonstrated similar *in vitro* and *in vivo* findings on two transplantable myelomonocytic leukemias in Balb/C mice. These animal findings suggest that not all leukemia cells have lost the capacity to differentiate Unfortunately no similar examples exist in humans. A large number of studies have been carried out in an attempt to induce human AML cells to differentiate *in vitro* in the presence of various forms of CSF [3 5 9 18, 23 36] The findings of these studies are in direct contrast to those noted above with murine leukemia cells. AML cells derived from the peripheral blood or bone marrow of most patients with AML form only small clusters or do not grow at all *in vitro* In a few patients, some colonies develop but whether these are derived from leukemic cell lines or from normal remaining hematopoietic stem cells has not been fully determined. Increasing the CSF stimulus to super maximal levels, washing of the cells vigorously prior to plating to remove possible inhibitors, does not alter the defective colony formation by human AML cells. The only system described in which colony formation by AML cells can be induced is that reported by *Dicke et al* [9] In this system, marrow cells are incubated overnight in the presence of phytohemagglutinin and then plated in an agar gel system free of phytohemagglutinin. In this system, large numbers of colonies develop which have been identified as being composed of leukemic cells on the basis of microscopic and karyotypic studies. Still not answered, however is whether differentiation occurs in AML cells stimulated in this fashion. Finally it is to be noted that bone marrow cells derived from patients with AML in complete remission appear to form normal colonies and clusters *in vitro* in the presence of appropriate stimuli. As relapse occurs and leukemic cells once again infiltrate the marrow colony formation falls and returns to pretreatment findings of no growth, or only cluster formation. These data suggest that remission in AML is a result of the return of normally responsive stem cell lines, as has been suggested for many years by clinical data.

The findings that human AML cells cannot be induced to *differentiate in vitro* in the majority of instances, regardless of the type and strength of the stimulus, suggests strongly an *intrinsic stem cell* defect with inability to respond to normal regulatory factors. This will come as no surprise to the majority of readers familiar to the demonstrated *karyotypic* abnormalities found in the cells of at least 50% of such patients. The major question to be answered in human AML is whether this defect can be defined and overcome The animal systems

described above suggest that such a goal is not untenable. In the long run this would seem to be the most hopeful approach to the treatment of this disorder but there are no clues at the present time in human systems as to how this can be accomplished. One of the most interesting approaches currently being pursued in our laboratory is the use of cell hybrids between normal human bone marrow derived granulocyte precursors and AML cells along with nuclear transfer experiments. It is hoped that such experiments may give us closer understanding of the nature of the regulatory defect in such cells.

Whether the concepts defined in AML can be applied to other human hematopoietic disorders involving abnormal stem cell proliferation, likewise remains a question for further exploration and experimentation. In the majority of instances, however it would appear that like AML, these diseases represent intrinsic stem cell abnormalities and not abnormalities in regulatory factors. In patients with polycythemia rubra vera, it has been demonstrated that erythropoietin levels in serum and urine are low for the level of red cell production. Likewise, it has been shown that bone marrow cells from patients with polycythemia rubra vera cultured in erythroid colony-forming assays form spontaneous red blood cell colonies in the absence of erythropoietin [1] In chronic myeloid leukemia, stem cell proliferation appears to be under normal control mechanisms, at least during the chronic stage of the disease. This is evidenced by the frequent finding of periodic oscillations in peripheral blood neutrophil counts and the fact that such cells respond to CSF and inhibitors *in vitro* [13] As blast crisis ensues, however the cells begin to lose their responsiveness and no longer form mature colonies *in vitro* once again suggesting the development of intrinsic stem cell abnormalities [29]

Our knowledge about the regulation of normal hematopoiesis is at best sketchy and tenuous. It may be that as these systems are further defined, the factors characterized and purified, the defect in stem cell proliferation can be overcome. This is a goal not yet foreseeable with our present knowledge.

References

1 Adamson, J W Fialkow P J Murphy S., *et al*.. Polycythemia vera. stem-cell and probable clonal origin of the disease. New Engl. J Med. 295 913–916 (1976).

2 Bradley T R. and Metcalf, D The growth of mouse bone marrow cells *in vitro*. Aust. J exp. Biol. med. Sci. *44* 287–300 (1966).

3 Brown, C. H. and Carbone, P P *In vitro* growth of normal and leukemic human bone marrow J natn. Cancer Inst. *46* 989–1000 (1971).

4 Broxmeyer H. E.; Smithyman, A.; Eger R. R., *et al*. Identification of lactoferrin as the granulocyte-derived inhibitor of colony-stimulating activity production. J exp. Med. *148*. 1052–1067 (1978).

5 Bull, J M., Rosenblum, A., and Carbone, P P *In vitro* culture studies in patients with acute myelocytic leukemia; in Robinson Hemopoiesis in culture. Publication No. (NIH) 72-205, pp. 335–341 (DHEW Washington 1973).

6 Chervenick, P A. and LoBuglio, A. F Human blood monocytes: stimulators of granulocyte and mononuclear formation *in vitro* Science *178* 164–166 (1972).

7 Chiyoda, S., Mizoguchi, H.; Asano, S., *et al*. Influence of leukaemic cells on the colony formation of human bone marrow cells *in vitro* II. Suppressive effects of leukaemic cell extracts. Br J Cancer *33* 379–384 (1976).

8 Cline, M. J and Golde, D. W Production of colony-stimulating activity by human lymphocytes. Nature, Lond. *48*. 703–704 (1974).

9 Dicke, K. A. Spitzer G and Ahearn, M. J Colony formation *in vitro* by leukaemic cells in acute myelogenous leukaemia with phytohaemagglutinin as stimulating factor Nature, Lond. 259 129–130 (1976)
10 Fibach, E. Gerassi, E., and Sachs, L. Induction of colony formation *in vitro* by human lymphocytes. Nature, Lond. 259 127–129 (1976)
11 Fibach, E. and Sachs, L. Control of normal differentiation of myeloid leukemic cells. IV Induction of differentiation by serum from endotoxin treated mice. J cell. Physiol. 83 177–186 (1974).
12 Fibach, E. and Sachs, L. Control of normal differentiation of myeloid leukemic cells. VII. Induction of differentiation to mature granulocytes in mass culture. J cell. Physiol. 86 221–230 (1975)
13 Gatti, R. A. Robinson, W A., Deinard, A. S., *et al* Cyclic leucocytosis in chronic myelogenous leukemia. new perspective on pathogenesis and therapy Blood 41 771–782 (1973).
14 Golde, D W and Cline, M J Endotoxin-induced release of colony-stimulating activity in man. Proc Soc. exp Biol. Med. 149 845–848 (1975).
15 Golde, D W and Cline, M J. Identification of the colony-stimulating cell in human peripheral blood. J clin. Invest. 52 2981–2983 (1972)
16 Goldman, J M. Th'ng, K. H., Catovsky D *et al.* Production of colony-stimulating factor by leukemic leukocytes. Blood 47 381–388 (1976).
17 Granström, M. Conditions influencing inhibitors of the colony-stimulating factor (CSF). Expl Cell Res. 87 307–312 (1974).
18 Greenberg, P L. Nichols, W and Schrier S L. Granulopoiesis in acute myeloid leukemia and preleukemia. New Engl J Med. 284 1225–1232 (1971).
19 Ichikawa, Y. Differentiation of a cell line of myeloid leukemia In Vincent, The nature of leukaemia, p. 187 (Australian Cancer Society Sydney 1972).
20 Mahmood, T and Robinson, W A. Granulocyte modulation of endotoxin-stimulated activity (CSA) production. Blood 51 879–887 (1978).
21 Mangalik, A. and Robinson, W A. The effect of serum from patients with acute granulocytic leukemia or granulocyte colony formation *in vitro* A search for inhibitors. Proc. Soc. exp Biol. Med. 141 515–518 (1972).
22 Maurer H. R. Chalones, specific regulators of eukaryote tissue growth, In Talwar, Regulation of growth and differentiated function in eukaryote cells, pp. 69–77 (Raven Press, New York 1975).
23 Metcalf, D. Hematopoietic colonies, *in vitro* cloning of normal and leukemic cells (Springer Berlin 1977)
24 Metcalf, D Chan, S. H., Gunz, F W *et al.* Colony stimulating factor and inhibitor levels in acute granulocytic leukemia. Blood 38 143–152 (1971).
25 Metcalf, D and Moore, M. A. S. Factors modifying stem cell proliferation of myelomonocytic leukemic cells *in vitro* and *in vivo*. J natn. Cancer Inst. 44 801–808 (1970).
26 Metcalf, D and Stanley E. R. Haematological effects in mice of partially purified colony stimulating factor (CSF) prepared from human urine Br J Haemat. 21 481–492 (1971).
27 Morris, T C. M., McNeill, T A., and Bridges, J M. Inhibition of normal human *in vitro* colony forming cells by cells from leukaemic patients. Br J Cancer 31 641–648 (1975).
28 Pluznik, D H. and Sachs, L. The cloning of normal 'mast' cells in tissue culture J cell. comp Physiol. 66. 319–324 (1965)
29 Rhodes, C. A., Robinson, W A., and Entringer M. A. Granulocyte colony formation *in* chronic granulocytic leukemia during *stable*, accelerated and blastic disease. Proc. Soc. exp. Biol. Med. 157 337–341 (1978)
30 Robinson, W A. and Entringer M A. Correlation of clinical disease and colony growth of human leukemic cells *in vitro* In Robinson, Hemopoiesis in culture. Publication No. (NIH)74-205 pp. 288–300 (DHEW Washington 1973).
31 Robinson, W A., Entringer M. A., Bolin, R. W *et al.* Bacterial stimulation and granulocyte inhibition of granulopoietic factor production. New Engl. J Med. 297 1129–1134 (1977).
32 Rozenszajn, L. A.; Shoham, D and Kalechman, L. Clonal proliferation of PHA stimulated human lymphocytes in soft agar culture. Immunology 29 1041–1055 (1975)

33 Rozenszajn, L. A., Sredni, B., Michlin, H., *et al.* Colony growth *in vitro* of B-lymphocytes. J Immunol. (1976).

34 Ruscetti, F W and Chervenick, P A. Release of colony-stimulating activity from thymus-derived lymphocytes. J clin. Invest. *55* 520–527 (1975).

35 Rytomaa, T and Kiviniemi, K. Control of granulocyte production. I Chalone and antichalone, two specific humoral regulators. Cell Tiss. Kinet. *1* 329–340 (1968).

36 Senn, J S., McCulloch, E. A., and Till, J E. Comparison of colony-forming ability of normal and leukaemic human bone marrow in cell culture. Lancet *II* 597–598 (1967).

37 Stephenson, J R., Axelrad, A. A., McLeod, D. L., *et al.* Induction of colonies of hemoglobin-synthesizing cells by erythropoietin *in vitro* Proc. natn. Acad. Sci. USA *68.* 1542–1546 (1971)

William A. Robinson, MD, PhD
Division of Medical Oncology
Department of Medicine,
University of Colorado Medical Center
Denver, CO 20262 (USA)

Announcements

Jean Julliard Prize

The 7th Jean Julliard Prize, which was established by the International Society of Blood Transfusion in memory of its first Secretary General, will be awarded during the 16th International Congress of Blood Transfusion, to be held in Montreal (Canada) on August 16–22, 1980.

The prize is reserved for scientists under 40 *years of age, in recognition of recently completed* scientific work on blood transfusion and related subjects.

In order to qualify candidates must forward six (6) copies of an unpublished manuscript or recently published papers including a curriculum vitae, to the Secretary General, Prof. C. Salmon, 53, boulevard Diderot, F-75571 Paris Cédex 12 (France) – *before March 15 1980*

The prize will be awarded during the congress. The value of the prize is 3 000 Swiss francs.

Further information is obtainable from the Secretary General.

European Organization for Research on Treatment of Cancer (EORTC)

Metastasis Group

A Conference on 'Metastasis: Clinical and Experimental' will be held at the Royal Institution, Albermarle Street, London, W1, April 21 23, 1980. There will be invited papers and free communications on metastatic potential, experimental models, host factors, therapy blood coagulability and clinicopathological aspects.

Secretariat: K. Hellmann, DM, Cancer Chemotherapy Department, Imperial Cancer Research Fund, Lincoln's Inn Fields, London, WC2 (England)

Author Index

(B) = Book Reviews

Subject Index

Compiled *G Booker*, Basel
(B) = Book reviews

Vol 63, 1980

Acta Haematologica

Founded 1948 by E. Meulengracht, K. Rohr and G. Rosenow
Continued by H. Lüdin (1960–1977)

Official Organ of the European Division of the International Society of Haematology
General Secretary J W Stewart, London

S. Karger Basel München Paris London New York Sydney

